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(54) **Human manganese superoxide dismutase DNA, its expression, method of recovering human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants, uses, compositions, and methods of treatment.**

(57) A double-stranded cDNA molecule which includes DNA encoding human manganese superoxide dismutase has been created. The sequence of one strand of a double-stranded DNA molecule which encodes human manganese superoxide dismutase has been discovered. Such molecules may be introduced in prokaryotic, e.g., bacterial, or eucaryotic, e.g., yeast or mammalian, cells and the resulting cells cultured or grown under suitable conditions so as to produce human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants which may then be recovered. By this invention, human manganese superoxide dismutase gene fragments from various plasmids may be ligated to yield a complete genomic human manganese superoxide dismutase gene fragment. Human manganese superoxide dismutase, analogs, or mutants may be used to catalyze the reduction of superoxide radicals, reduce reperfusion injury, prolong the survival time of isolated organs, or treat inflammations.

The invention also concerns a method of producing enzymatically active human manganese superoxide dismutase compounds in a bacterial cell which contains and is capable of expressing a DNA sequence encoding the superoxide dismutase compound by maintaining the bacterial cell under suitable conditions and in a suitable production medium. The production medium is supplemented with an amount of Mn^{++} so that the concentration of Mn^{++} in the medium is greater than about 2 ppm. Genomic manganese superoxide dismutase DNA should also be capable of expression in eucaryotic cells under suitable conditions.

This invention also concerns a method of recovering purified enzymatically active manganese superoxide dismutase, analogs or mutants from bacterial cells. It should also be possible to recover manganese SOD from

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genomic manganese superoxide dismutase DNA expressed in eucaryotic cells using similar methods.

The invention further concerns uses of the human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants, various new analogs, compositions of the compounds and methods of treating various disorders using these compositions.

HUMAN MANGANESE SUPEROXIDE DISMUTASE DNA; ITS EXPRESSION; METHOD OF RECOVERING HUMAN MANGANESE SUPEROXIDE DISMUTASE, HUMAN MANGANESE SUPEROXIDE DISMUTASE ANALOGS OR HUMAN MANGANESE SUPEROXIDE DISMUTASE MUTANTS; USES; COMPOSITIONS; AND METHODS OF TREATMENT

This application is a continuation-in-part of U.S. Serial No. 032,734, filed March 27, 1987, which is a continuation-in-part of U.S. Serial No. 907,051, filed September 12, 1986, which in turn is a continuation-in-part of U.S. Serial No. 801,090, filed November 22, 1985, the contents of which applications are hereby incorporated by reference into the subject application.

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BACKGROUND OF THE INVENTION

10 Throughout this application, various publications are referenced by arabic numerals within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of art as known to those skilled therein as of the date of the invention described and claimed herein.

15 Superoxide dismutase (SOD) and the phenomenon of oxygen free radicals (O_2^-) was discovered in 1968 by McCord and Fridovich (1). Superoxide radicals and other highly reactive oxygen species are produced in every respiring cell as by-products of oxidative damage to a wide variety of macromolecules and cellular components (for review see 2,3). A group of metalloproteins known as superoxide dismutases catalyze the oxidation-reduction reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ and thus provide a defense mechanism against oxygen toxicity.

20 There are several known forms of SOD containing different metals and different proteins. Metals present in SOD include iron, manganese, copper and zinc. All of the known forms of SOD catalyze the same reaction. These enzymes are found in several evolutionary groups. Superoxide dismutases containing iron are found primarily in prokaryotic cells. Superoxide dismutases containing copper and zinc has been found in virtually all eucaryotic organisms (4). Superoxide dismutases containing manganese have been found in 25 organisms ranging from microorganisms to man.

Since every biological macromolecule can serve as a target for the damaging action of the abundant superoxide radical, interest has evolved in the therapeutic potential of SOD. The scientific literature suggests that SOD may be useful in a wide range of clinical applications. These include prevention of oncogenesis and of tumor promotion, and reduction of the cytotoxic and cardiotoxic effects of anticancer 30 drugs (10), protection of ischemic tissues (12) and protection of spermatozoa (13). In addition, there is interest in studying the effect of SOD on the aging process (14).

The exploration of the therapeutic potential of human SOD has been limited mainly due to its limited availability.

Superoxide dismutase is also of interest because of its anti-inflammatory properties (11). Bovine-derived 35 superoxide dismutase (orgotein) has been recognized to possess anti-inflammatory properties and is currently marketed in parts of Europe as a human pharmaceutical. It is also sold in the United States as a veterinary product, particularly for the treatment of inflamed tendons in horses. However, supplies of orgotein are limited. Prior techniques involving recovery from bovine or other animal cells have serious limitations and the orgotein so obtained may produce allergic reactions in humans because of its non-40 human origin.

Copper zinc superoxide dismutase (CuZn SOD) is the most studied and best characterized of the various forms of superoxide dismutase.

Human CuZn SOD is a dimeric metalloprotein composed of identical non-covalently linked subunits, each having a molecular weight of 16,000 daltons and containing one atom of copper and one of zinc (5). 45 Each subunit is composed of 153 amino acids whose sequence has been established (6,7).

The cDNA encoding human CuZn superoxide dismutase has been cloned (8). The complete sequence of the cloned DNA has also been determined (9). Moreover, expression vectors containing DNA encoding superoxide dismutase for the production and recovery of superoxide dismutase in bacteria have been described (24,25). The expression of a superoxide dismutase DNA and the production of SOD in yeast has 50 also been disclosed (26).

Recently, the CuZn SOD gene locus on human chromosome 21 has been characterized (27) and recent developments relating to CuZn superoxide dismutase have been summarized (28).

Much less is known about manganese superoxide dismutase (MnSOD). The manganese superoxide dismutase of E. coli K-12 has recently been cloned and mapped (22). Barra et al. disclose a 196 amino acid sequence for the manganese superoxide dismutase polypeptide isolated from human liver cells (19). Prior art disclosures differ, however, concerning the structure of the manganese superoxide dismutase molecule,
5 particularly whether it has two or four identical polypeptide subunits (19,23). It is clear, however, that the manganese superoxide dismutase polypeptide and the CuZn SOD polypeptide are not homologous (19). The amino acid sequence homologies of MnSODs and FeSOD from various sources have also been compared (18).

Baret et al. disclose in a rat model that the half life of human manganese superoxide dismutase is
10 substantially longer than the half-life of human copper SOD; they also disclose that in the rat model, human manganese superoxide dismutase and rat copper SOD are not effective as anti-inflammatory agents whereas bovine copper SOD and human copper SOD are fully active (20).

McCord et al. disclose that naturally occurring human manganese superoxide dismutase protects human phagocytosing polymorphonuclear (PMN) leukocytes from superoxide free radicals better than
15 bovine or porcine CuZn superoxide dismutase in "in vitro" tests (21).

The present invention concerns the preparation of a cDNA molecule encoding the human manganese superoxide dismutase polypeptide or an analog or mutant thereof.

It is also directed to inserting this cDNA into efficient bacterial expression vectors, to producing human manganese superoxide dismutase polypeptide, analog, mutant and enzyme in bacteria, to recovering the
20 bacterially produced human manganese superoxide dismutase polypeptide, analog, mutant or enzyme. This invention is also directed to the human manganese superoxide dismutase polypeptides, analogs, or mutants thereof so recovered and their uses.

This invention further provides a method for producing enzymatically active human manganese superoxide dismutase in bacteria, as well as a method for recovering and purifying such enzymatically
25 active human MnSOD.

The present invention also relates to a DNA molecule encoding the human manganese superoxide dismutase gene. It is also directed to inserting the DNA into mammalian cells to produce manganese superoxide dismutase polypeptide, analog, mutant and enzyme.

The present invention also relates to using human manganese superoxide dismutase or analogs or
30 mutants thereof to catalyze the reduction of superoxide radicals to hydrogen peroxide and molecular oxygen. In particular, the present invention concerns using bacterially produced manganese superoxide dismutase or analogs or mutants thereof to reduce reperfusion injury following ischemia and prolong the survival period of excised isolated organs.

The invention further concerns the use of bacterially produced human manganese superoxide dis-
35 mutase, human manganese superoxide dismutase, or human manganese superoxide dismutase mutants to treat inflammations. In particular, the invention relates to the use of human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants to treat disorders associated with the generation of oxygen free radicals in quantities to cause undesirable symptoms. Such disorders treated include: (1) synovial inflammation induced by bacterial
40 lipopolysaccharide endotoxin (LPS); (2) inflammation caused by adjuvant-induced arthritis; or (3) bleomycin-induced lung fibrosis. The invention further relates to methods of administering the human manganese superoxide dismutase, the human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants and to amounts and doses of human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants.

45 The invention also concerns various human manganese superoxide dismutase analogs in which at least one of the human manganese superoxide dismutase polypeptides differs from naturally-occurring human manganese superoxide dismutase by the absence of one or more amino acids at the N-terminus. These analogs may be used in compositions and the compositions may be used in methods of treating various disorders by alleviating the undesirable symptoms associated with the generation of oxygen free radicals.

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SUMMARY OF THE INVENTION

A DNA molecule which includes cDNA encoding the human manganese superoxide dismutase polypep-
55 tide or analog or mutant thereof has been isolated from a human T-cell library. The nucleotide sequence of a double-stranded DNA molecule which encodes human manganese superoxide dismutase polypeptide or analog or mutant thereof has been discovered. The sequence of one strand encoding the polypeptide or analog thereof is shown in Figure 1 from nucleotide 115 downstream to nucleotide 708 inclusive. Other

sequences encoding the analog or mutant may be substantially similar to the strand encoding the polypeptide. The nucleotide sequence of one strand of a double stranded DNA molecule which encodes a twentyfour (24) amino acid prepeptide is also shown in Figure 1, from nucleotides number 43 through 114, inclusive.

5   The double-stranded cDNA molecule or any other double-stranded DNA molecule which contains a nucleotide strand having the sequence encoding the human manganese superoxide dismutase polypeptide or analog or mutant thereof may be incorporated into a cloning vehicle such as a plasmid or virus. Either DNA molecule may be introduced into a cell, either prokaryotic, e.g., bacterial, or eucaryotic, e.g., yeast or mammalian, using known methods, including but not limited to methods involving cloning vehicles
10 containing either molecule.

    Preferably the cDNA or DNA encoding the human manganese superoxide dismutase polypeptide or analog or mutant thereof is incorporated into a plasmid, e.g., pMSE-4 or pMSΔRB4, and then introduced into a suitable host cell where the DNA can be expressed and the human manganese superoxide dismutase (hMnSOD) polypeptide or analog or mutant thereof produced. Preferred host cells include Escherichia coli,
15 in particular E. coli A4255 and E. coli A1645. The plasmid pMSE-4 in E. coli strain A4255 has been deposited with the American Type Culture Collection under ATCC Accession No. 53250. The plasmid pMSΔRB4 may be obtained as shown in Figure 4 and described in the Description of the Figures.

    Cells into which such DNA molecules have been introduced may be cultured or grown in accordance with methods known to those skilled in the art under suitable conditions permitting transcription of the DNA
20 into mRNA and expression of the mRNA as protein. The resulting manganese superoxide dismutase protein may then be recovered.

    Veterinary and pharmaceutical compositions containing human manganese superoxide dismutase or analogs or mutants thereof and suitable carriers may also be prepared. This human manganese superoxide dismutase or analogs or mutants may be used to catalyze the following reaction:

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$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
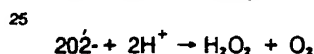
and thereby reduce cell injury caused by superoxide radicals.

    More particularly, these enzymes or analogs or mutants thereof may be used to reduce injury caused
30 by reperfusion following ischemia, increase the survival time of excised isolated organs, or treat inflammations.

    This invention is directed to a method of producing enzymatically active human manganese superoxide dismutase or an analog or mutant thereof in a bacterial cell. The bacterial cell contains and is capable of expressing a DNA sequence encoding the manganese superoxide dismutase or analog or mutant thereof.
35 The method comprises maintaining the bacterial cell under suitable conditions and in a suitable production medium. The production medium is supplemented with an amount of Mn^{++} so that the concentration of Mn^{++} available to the cell in the medium is greater than about 2 ppm.

    In a preferred embodiment of the invention the bacterial cell is an Escherichia coli cell containing a plasmid which contains a DNA sequence encoding for the human manganese superoxide dismutase
40 polypeptide e.g. pMSE-4 or pMSΔRB4 in E. coli strain A4255. The concentration of Mn^{++} in the production medium ranges from about 50 to about 1500 ppm, with concentrations of 150 and 750 ppm being preferred.

    This invention also concerns a method of recovering manganese superoxide dismutase analog or mutant thereof from bacterial cells which contain the same. The cells are first treated to recover a protein fraction containing proteins present in the cells including human manganese superoxide dismutase or
45 analog or mutant thereof and then the protein fraction is treated to recover human manganese superoxide dismutase or analog or mutant thereof. In a preferred embodiment of the invention, the cells are first treated to separate soluble proteins from insoluble proteins and cell wall debris and the soluble proteins are recovered. The soluble proteins are then treated to separate, e.g. precipitate, a fraction of the soluble proteins containing the hMnSOD or analog or mutant thereof and the fraction containing the hMnSOD or
50 analog or mutant is recovered. The recovered fraction of soluble proteins is then treated to separately recover the human manganese superoxide dismutase or analog thereof.

    A more preferred embodiment of the invention concerns a method of recovering human manganese superoxide dismutase or analog or mutant thereof from bacterial cells which contain human manganese superoxide dismutase or analog or mutant thereof. The method involves first isolating the bacterial cells
55 from the production medium and suspending them in suitable solution having a pH of about 7.0 to 8.0. The cells are then disrupted and centrifuged and the resulting supernatant is heated for about 30 to 120 minutes at a temperature between 55 and 65°C, preferably for 45-75 minutes at 58-62°C and more preferably for 1 hour at 60°C and then cooled to below 10°C, preferably to 4°C. Any precipitate which forms is to be

removed e.g. by centrifugation, and the cooled supernatant is dialyzed against an appropriate buffer e.g. 2 mM potassium phosphate buffer having a pH of about 7.8. Preferably, the dialysis is by ultrafiltration using a filtration membrane smaller than 30K. Simultaneously with or after dialysis the cooled supernatant optionally may be concentrated to an appropriate convenient volume e.g. 0.03 of its original volume. The

5 retentate is then eluted on an anion exchange chromatography column with an appropriate buffered solution e.g., a solution of at least 20 mM potassium phosphate buffer having a pH of about 7.8. The fractions of eluent containing superoxide dismutase are collected, pooled and dialyzed against about 40 mM potassium acetate, pH 5.5. The dialyzed pooled fractions are then eluted through a cation exchange chromatography column having a linear gradient of about 40 to about 200 mM potassium acetate and a pH of 5.5. The peak

10 fractions containing the superoxide dismutase are collected and pooled. Optionally the pooled peak fractions may then be dialyzed against an appropriate solution e.g. water or a buffer solution of about 10 mM potassium phosphate buffer having a pH of about 7.8.

The invention also concerns purified enzymatically active human manganese superoxide dismutase or analogs thereof e.g. met-hMnSOD, or mutants produced by the methods of this invention.

15 The present invention also relates to a DNA molecule encoding the human manganese superoxide dismutase gene. The nucleotide sequence of the exon coding regions of one strand of the manganese superoxide dismutase gene is shown in Figure 6. The DNA encoding the manganese superoxide dismutase gene may be incorporated into a cloning vehicle such as a plasmid or a virus. The DNA or the cloning vehicle may be introduced into eucaryotic cells using known methods. The DNA encoding the human

20 manganese superoxide dismutase gene is encoded in the plasmids pMSG11-1, pMSG4 and pMSG-1b. The eucaryotic cells which are transformed are preferably mammalian cell lines such as the human HeLa cell line or the mouse L cell line. Another aspect of this invention is the production of the human manganese superoxide dismutase polypeptide, analog, mutant or enzyme by growing the cells of this invention in suitable medium under suitable conditions.

25 The invention also relates to a double-stranded DNA molecule which encodes human manganese superoxide dismutase, human manganese superoxide dismutase analog and the coding strand of which has the nucleotide sequence shown in Figure 6. The invention further relates to a double-stranded molecule which encodes human manganese superoxide dismutase mutant and the coding strand of which has a nucleotide sequence which varies from the nucleotide sequence shown in Figure 6 by one or more

30 nucleotides.

The present invention is further concerned with eucaryotic cells in which the DNA sequence encoding human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is expressed; and with eucaryotic cells wherein the cell has been transformed with a plasmid comprising the DNA sequence encoding human manganese superoxide

35 dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant. This DNA sequence may be present in single or multiple copies. The DNA sequence encoding human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is derived from a fragment of human genomic DNA containing the human manganese superoxide dismutase gene. In addition, the DNA sequence encoding human

40 manganese superoxide dismutase is regulated by the natural regulatory elements of human genomic manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant. An example of a DNA sequence coding for the human manganese superoxide dismutase, the analog or mutant and coding for the native regulatory elements regulating the gene is shown in the DNA sequence of Figure 6.

45 The eucaryotic cell expressing the genomic DNA sequence of human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is a mammalian cell. Examples of mammalian cells which may express the genomic DNA sequence are the human HeLa cell and mouse L cells.

Methods for producing human manganese superoxide dismutase, human manganese superoxide

50 dismutase analog or human manganese superoxide dismutase mutant which comprise growing the eucaryotic cells described above in a suitable medium under suitable conditions permitting production of the human manganese superoxide dismutase, the analog or the mutant thereof and permitting the recovery of the resulting human manganese superoxide dismutase, the analog or the mutant are also contemplated by this invention. Human manganese superoxide dismutase polypeptides, human manganese superoxide

55 dismutase analogs and human manganese superoxide dismutase mutants produced by this method are also contemplated by this invention.

The invention is also concerned with human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants used to treat inflamma-

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tion, in particular, synovial inflammation, induced by lipopolysaccharide endotoxin (LPS); inflammation caused by adjuvant-induced arthritis; and bleomycin-induced lung fibrosis. The use of manganese superoxide dismutase, manganese superoxide dismutase analogs and manganese superoxide dismutase mutants to treat these various disorders is discussed in greater detail in the Exam ples which follow. The invention also contemplates amounts and doses of the human manganese superoxide dismutase, analogs or mutants thereof used to alleviate symptoms and treat disorders and to methods of administration.

The invention is further concerned with human manganese superoxide dismutase analogs in which one of the human manganese superoxide dismutase polypeptides differs from naturally-occurring human manganese superoxide dismutase by the absence of one or more amino acids from the N-terminus.

Compositions comprising human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants and a suitable carrier are also contemplated by this invention. These compositions may be used for treating any disorder for which the naturally-occurring superoxide dismutase may be used and for alleviating the undesirable symptoms associated with the generation of oxygen free radicals. These disorders and symptoms include, but are not limited to, reperfusion injury following ischemia or organ transplants, inflammations, bronchial pulmonary dysplasia, fibrosis caused by radiation treatment, inflammation caused by Crohn's disease or inflammation caused by collitis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. The Sequence of Human Manganese Superoxide Dismutase cDNA
Figure 1 shows the nucleotide sequence of one strand of a double-stranded DNA molecule encoding the human manganese superoxide dismutase as well as the 198 amino acid sequence of human manganese superoxide dismutase corresponding to the DNA sequence. Figure 1 also shows the nucleotide sequence of one strand of a double stranded DNA molecule encoding a prepeptide to the mature human manganese superoxide dismutase consisting of twenty-four amino acids and the amino acid sequence corresponding to that DNA sequence. Also shown are the 5' and 3' untranslated sequences.

Figure 2. Construction of pMSE-4: Human Manganese Superoxide Dismutase Expression Plasmid
Plasmid pMS8-4, containing manganese superoxide dismutase on an EcoRI (R₁) insert, was digested to completion with Ndel and Narl restriction enzymes. The large fragment was isolated and ligated with a synthetic oligomer as depicted in Figure 2. The resulting plasmid, pMS8-NN contains the coding region for the mature MnSOD, preceded by an ATG initiation codon. The above plasmid was digested with EcoRI, ends were filled in with Klenow fragment of Polymerase I and further cleaved with Ndel. The small fragment harboring the manganese superoxide dismutase gene was inserted into pSODα13 which was treated with Ndel and Stul. pSODα-13 may be obtained as described in pending co-assigned U.S. Patent Application Serial No. 644,245, filed August 27, 1984 which is hereby incorporated by reference. This generated plasmid pMSE-4 containing the manganese superoxide dismutase coding region preceded by the cII ribosomal binding site and under the control of λ P$_L$ promoter. Plasmid pMSE-4 has been deposited with the American Type Culture Collection under ATCC Accession No. 53250.

Figure 3. Effect of Mn^{++} Concentration on the Activity of Superoxide Dismutase Produced in E. Coli
The chart in Figure 3 shows the correlation between the specific activity in units/mg of recombinant soluble manganese superoxide dismutase produced by E. coli strain A4255 containing plasmid pMSE-4 under both non-induction (32°C) and induction (42°C) conditions, and the concentration of Mn^{++} (parts per million) in the growth medium.

Figure 4. Construction of pMSΔRB4: Human Manganese Superoxide Dismutase Expression Plasmid
Tet^R expression vector, pΔRB, was generated from pSODβ,T-11 by complete digestion with EcoRI followed by partial cleavage withBamHI restriction enzymes. pSODβ,T-11 has been deposited with the American Type Culture Collection (ATCC) under Accession No. 53468. The digested plasmid was ligated with synthetic oligomer

5' - AATTCCCGGGTCTAGATCT - 3'

3' -   GGGCCCAGATCTAGACTAG - 5'

resulting in pΔRB containing the λ P$_L$ promoter.

(Freund's adjuvant) - induced arthritis. Extent of arthritis is expressed as the increase in paw volume following adjuvant injection. The increase in paw volume is calculated relative to the paw volume 14 days after adjuvant injection; this is the period of immunologically-induced chronic arthritis. Manganese Superoxide Dismutase and CuZn SOD were administered in doses according to body weight (b.w.) to adjuvant

5 treated rats beginning 14 days post-adjuvant injection. Seven groups of rats were used in the study (6 rats per group). All animals were injected on Day 1 with 50 μl Freund's adjuvant containing 10 mg/ml killed Mycobacterium Tuberculosis. On days 14-20, the following treatments were carried out: group 1 (control) rats received no treatment; group 2 rats received 12.5 mg/kg b.w. doses of manganese superoxide dismutase each day for 7 days; group 3 rats received 25 mg/kg b.w. of manganese superoxide dismutase

10 each day for 7 days; group 4 rats received 12.5 mg/kg b.w. of CuZn SOD each day for 7 days; group 5 rats received 25 mg/kg b.w. of CuZn SOD each day for 7 days; group 6 rats received 50 mg/kg b.w. of manganese superoxide dismutase every second (alternate) day, that is 3 manganese superoxide dismutase treatments on days 1, 3, 5; group 7 rats received 50 mg/kg b.w. of CuZn SOD every second (alternate) day (days 1, 3, 5).

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At day 21 the animals were examined for amount of paw swelling relative to day 14. Statistical analysis was carried out according to Student t-test.

Figure 13. Therapeutic Effect of Recombinant Manganese Superoxide Dismutase in Treatment of Bleomycin Induced Lung Fibrosis

20 Bar graph depicting the therapeutic effect of manganese superoxide dismutase on Bleomycin-induced lung fibrosis.

The degree of Bleomycin-induced lung fibrosis is expressed in two ways: (a) the ratio of lung weight to body weight; (2) the amount of hydroxyproline per lung (μg/g wet weight).

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Three groups of rats were used in this study (15 rats per group): Group 1 rats (negative control) were sham-treated and received only saline. Group 2 rats were treated with 1.5 units per rat (= 1.5 mg) of Bleomycin administered intra-tracheally on Day 1. Group 3 rats (positive control) were given Bleomycin (1.5 units intra-tracheally per rat) on day 1, followed by daily subcutaneous injections of manganese superoxide dismutase

30 (50 mg/kg body weight) on days 1-7.

Three weeks after the start of the study, the surviving animals were sacrificed and their lungs were excised. The wet weights of each lung was determined and the hydroxyproline content of each lung was assayed (using standard methods as described in Example 14). The open bars represent the ratio of lung weight to

35 body weight and the shaded bars represent the content of hydroxyproline per wet tissue weight. The brackets represent the standard error of the mean (S.E.M.).

Figure 14. Effect of Daily Subcutaneous Injection of Recombinant Human Manganese Superoxide Dismutase on Bleomycin Induced Lung Fibrosis

Three groups of rats (100-120 grams body weight) (15 rats/group) were used. One group was given

40 bleomycin (1.5 units/rat) intratracheally on Day 1. The second group received bleomycin as in group I as well as recombinant human manganese superoxide dismutase (50 mg/μg) subcutaneously, once daily, for 7 days. (The recombinant human manganese superoxide dismutase was administered two hours before the administration of the bleomycin. A third group (negative control) was given a single injection of saline intratracheally on Day 1.

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After three weeks, all surviving animals were sacrificed, their lungs were excised and the lung collagen content (expressed as hydroxy-proline) was determined. The results are shown in Figure 14.

Figure 15. Effect of Manganese Superoxide Dismutase Administration Frequency on Bleomycin-Induced Lung Fibrosis

50 The experimental details are essentially the same as described in Figure 14, however, the frequency of administration of manganese superoxide dismutase was changed. The recombinant human manganese superoxide dismutase was administered by a single injection subcutaneously on Day 1 or administered subcutaneously every other day (Days 1, 3, and 5). Also, the effectiveness of the recombinant CuZn SOD analog in preventing lung fibrosis was analyzed. The results are shown in Figure 15.

55 Figure 16. Effect of Intraperitoneal Administered Manganese Superoxide Dismutase on Bleomycin-Induced Lung Fibrosis

The experimental details are essentially the same as described in the description of Figure 14, however, the manganese superoxide dismutase or CuZn SOD analog was administered via the intraperitoneal route

rather than subcutaneously. The manganese superoxide dismutase or CuZn SOD was administered as a single dose on every other day as described in the description to Figure 15. The results are shown in Figure 16.

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DETAILED DESCRIPTION OF THE INVENTION

A double-stranded DNA molecule which includes cDNA encoding human manganese superoxide dismutase polypeptide or an analog or mutant thereof has been isolated from a human T-cell DNA library.

10 The nucleotide sequence of a double-stranded DNA molecule which encodes human manganese superoxide dismutase polypeptide or an analog or mutant thereof has been discovered. The sequence of one strand of DNA molecule encoding the human manganese superoxide dismutase polypeptide or analog thereof is shown in Figure 1 and includes nucleotides numbered 115 to 708 inclusive. The sequence of one strand encoding human manganese superoxide dismutase analog or mutant is substantially similar to the

15 strand encoding the human manganese superoxide dismutase polypeptide. The nucleotide sequence of the prepeptide of human manganese superoxide dismutase is also shown in Figure 1. Nucleotides numbered 43 through 114 inclusive code for this prepeptide.

The methods of preparing the cDNA and of determining the sequence of DNA encoding the human manganese superoxide dismutase polypeptide, analog or mutant thereof are known to those skilled in the

20 art and are described more fully hereinafter. Moreover, now that the DNA sequence which encodes the human manganese superoxide dismutase has been discovered, known synthetic methods can be employed to prepare DNA molecules containing portions of this sequence.

Conventional cloning vehicles such as plasmids, e.g., pBR322, viruses or bacteriophages, e.g., can be modified or engineered using known methods so as to produce novel cloning vehicles which contain cDNA

25 encoding human manganese superoxide dismutase polypeptide, or analogs or mutants thereof. Similarly, such cloning vehicles can be modified or engineered so that they contain DNA molecules, one strand of which includes a segment having the sequence shown in Figure 1 for human manganese superoxide dismutase polypeptide or segments substantially similar thereto. The DNA molecule inserted may be made by various methods including enzymatic or chemical synthesis.

30 The resulting cloning vehicles are chemical entities which do not occur in nature and may only be created by the modern technology commonly described as recombinant DNA technology. Preferably the cloning vehicle is a plasmid, e.g. pMSE-4 or pMSΔRB4. These cloning vehicles may be introduced in cells, either prokaryotic, e.g., bacterial (Escherichia coli, B.subtilis, etc.) or eucaryotic, e.g., yeast or mammalian using techniques known to those skilled in the art, such as transformation, transfection and the like. The

35 cells into which the cloning vehicles are introduced will thus contain cDNA encoding human manganese superoxide dismutase polypeptide or analog or mutant thereof if the cDNA was present in the cloning vehicle or will contain DNA which includes a strand, all or a portion of which has the sequence for human manganese superoxide dismutase polypeptide shown in Figure 1 or sequence substantially similar thereto if such DNA was present in the cloning vehicle.

40 Escherichia coli are preferred host cells for the cloning vehicles of this invention. A presently preferred auxotrophic strain of E. coli is A1645 which has been deposited with the American Type Culture Collec tion in Rockville, Maryland, U.S.A. containing plasmid pApoE-Ex2, under ATCC Accession No. 39787. All deposits with the American Type Culture Collection referred to in this application were made pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms.

45 A1645 was obtained from A1637 by selection for Gal$^+$ (ability to ferment galactose) as well as loss of tetracycline resistance. It still contains elements of phage λ. Its phenotype is C600 r$^-$m$^+$ gal$^+$ thr$^-$ leu$^-$ lacZ$^-$ bl (λ cl857 ΔH1 ΔBam H1 N$^+$).

A1637 was obtained from C600 by inserting transposon containing tetracycline resistance gene into the galactose operon as well as elements of phage λ including those elements responsible for cl repressor

50 synthesis. C600 is available from the American Type Culture Collection, as ATCC Accession No. 23724.

Prototrophic strains of Escherichia coli which enable high level polypeptide expression even when grown in a minimal media are even more preferred as hosts for expression of genes encoding manganese superoxide dismutase. One presently preferred prototrophic strain is A4255. Strain A4255 containing the plasmid pMSE-4 has been deposited with the American Type Culture Collection under ATCC Accession No.

55 53250.

The resulting cells into which DNA encoding human manganese superoxide dismutase polypeptide or analog or mutant thereof has been introduced may be treated, e.g. grown or cultured as appropriate under suitable conditions known to those skilled in the art, so that the DNA directs expression of the genetic

information en coded by the DNA, e.g. directs expression of the hMnSOD polypeptide or analog or mutant thereof, and the cell expresses the human manganese superoxide dismutase polypeptide or analog or mutant thereof which may then be recovered.

As used throughout this specification, the term "superoxide dismutase" (SOD) means an enzyme or a polypeptide acting upon superoxide or oxygen-free radicals as receptors, or which catalyze the following dismutation reaction:

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$
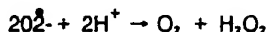
The term "manganese superoxide dismutase" (MnSOD) as used herein means any superoxide dismutase molecule containing the element manganese, in any of its chemical forms.

The term "human manganese superoxide dismutase polypeptide" as used herein means a polypeptide of 198 amino acids a portion of the amino acid sequence of which is shown in Figure 1; the N-terminus of the sequence is the lysine encoded by nucleotides 115-117 of Figure 1 and the COOH terminus of the sequence is the lysine encoded by nucleotides 706-708 of Figure 1.

The term "polypeptide manganese complex" as used herein means a molecule which includes a human manganese superoxide dismutase polypeptide in a complex with manganese in any of its chemical forms and which has the enzymatic activity of naturally-occurring human manganese superoxide dismutase.

The term "human manganese superoxide dismutase" as used herein means a molecule which includes at least two human manganese superoxide dismutase polypeptides in a complex with manganese in any of its chemical forms and which has the enzymatic activity of naturally-occurring human manganese superoxide dismutase.

The term "human manganese superoxide dismutase polypeptide analog" as used herein means a polypeptide which includes a human manganese superoxide dismutase polypeptide to either or both ends of which one or more additional amino acids are attached or removed.

The term "polypeptide manganese complex analog" as used herein means a molecule which includes a polypeptide manganese complex, the polypeptide portion of which includes one or more additional amino acids attached or removed from it at either or both ends.

The term "human manganese superoxide dismutase analog" as used herein means a molecule that includes at least two polypeptides at least one of which is human manganese superoxide dismutase polypeptide analog, in a complex with manganese in any of its chemical forms, and which has the enzymatic activity of naturally-occurring human manganese superoxide dismutase.

The term "human manganese superoxide dismutase polypeptide mutant" as used herein means a polypeptide having an amino acid sequence substantially identical to that of the human manganese superoxide dismutase polypeptide but differing from it by one or more amino acids.

The term "polypeptide manganese complex mutant" means a molecule which includes a human manganese superoxide dismutase polypeptide mutant in a complex with manganese in any of its chemical forms and which has the enzymatic activity of manganese superoxide dismutase.

The term "human manganese superoxide dismutase mutant" as used herein means a molecule which includes at least two polypeptides at least one of which polypeptides is a human manganese superoxide dismutase polypeptide mutant in a complex with manganese in any of its chemical forms and which has the enzymatic activity of naturally-occurring human manganese superoxide dismutase.

The mutants of human manganese superoxide dismutase polypeptide and human manganese superoxide dismutase which are included as a part of this invention may be prepared by mutating the DNA sequence shown in Figure 1, the N-terminus of which. sequence is the lysine encoded by nucleotides 115-117 and the COOH terminus of which sequence is encoded by nucleotides 706-708.

The DNA may be mutated by methods known to those of ordinary skill in the art, e.g. Bauer et al., Gene 37: 73-81 (1985). The mutated sequence may be inserted into suitable expression vectors as described herein, which are introduced into cells which are then treated so that the mutated DNA directs expression of the human manganese superoxide dismutase polypeptide mutants and the human manganese superoxide dismutase mutants.

The enzymatically active form of human manganese superoxide dismutase is believed to be a protein having at least two, and possibly four, identical subunits, each of which has approximately 198 amino acids in the sequence shown in Figure 1 for human manganese superoxide dismutase, the N-terminus of the sequence being the lysine encoded by nucleotides 115-117 of Figure 1 and the COOH terminus of the sequence being the lysine encoded by nucleotides 706-708 of Figure 1.

Human manganese superoxide dismutase or analogs or mutants thereof may be prepared from cells

into which DNA or cDNA encoding human manganese superoxide dismutase, or its analogs or mutants have been introduced. This human manganese superoxide dismutase or analogs or mutants may be used to catalyze the dismutation or univalent reduction of the superoxide anion in the presence of protons to form hydrogen peroxide as shown in the following equation:

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{human MnSOD}} H_2O_2 + O_2$$
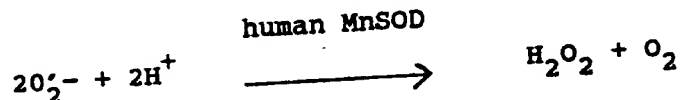
Veterinary and pharmaceutical compositions may also be prepared which contain effective amounts of human manganese superoxide dismutase or one or more human manganese superoxide dismutase analogs or mutant and a suitable carrier. Such carriers are well-known to those skilled in the art. The human manganese superoxide dismutase or analog or mutant thereof may be administered directly or in the form of a composition to the animal or human subject, e.g., to treat a subject afflicted by inflammations or to reduce injury to the subject by oxygen-free radicals on reperfusion following ischemia or organ transplantation. The human manganese superoxide dismutase or analog or mutant may also be added directly or in the form of a composition to the perfusion medium of an isolated organ, to reduce injury to an isolated organ by oxygen-free radicals on perfusion after excision, thus prolonging the survival period of the organ. Additionally, the human manganese superoxide dismutase or analog or mutant thereof may be used to reduce neurological injury on reperfusion following ischemia and to treat bronchial pulmonary dysplasia.

A method of producing enzymatically active human manganese superoxide dismutase or an analog or mutant thereof in a bacterial cell has also been discovered. The bacterial cell contains and is capable of expressing a DNA sequence encoding the human manganese superoxide dismutase or analog or mutant thereof. The method involves maintaining the bacterial cell under suitable conditions and in a suitable production medium. The production medium is supplemented with an amount of Mn^{++} so that the concentration of Mn^{++} in the medium is greater than about 2 ppm.

The bacterial cell can be any bacterium in which a DNA sequence encoding human manganese superoxide dismutase has been introduced by recombinant DNA techniques. The bacterium must be capable of expressing the DNA sequence and producing the protein product. The suitable conditions and production medium will vary according to the species and strain of bacterium.

The bacterial cell may contain the DNA sequence encoding the superoxide dismutase or analog in the body of a vector DNA molecule such as a plasmid. The vector or plasmid is constructed by recombinant DNA techniques to have the sequence encoding the SOD incorporated at a suitable position in the molecule.

In a preferred embodiment of the invention the bacterial cell is an Escherichia coli cell. A preferred auxotrophic strain of E. coli is A1645. A preferred prototrophic strain of E. coli is A4255 The E. coli cell of this invention contains a plasmid which encodes for human manganese superoxide dismutase or an analog or mutant thereof.

In a preferred embodiment of this invention, the bacterial cell contains the plasmid pMSE-4. A method of constructing this plasmid is described in the Description of the Figures and the plasmid itself is described in Example 2. This plasmid has been deposited with the ATCC under Accession No. 43250.

In another preferred embodiment of this invention, the bacterial cell contains the plasmid pMSΔRB4. A method of constructing this plasmid is described in the Description of the Figures and the plasmid itself is described in Example 5. This plasmid may be constructed from pSODβ,T-11 which has been deposited with the American Type Culture Collection under Accession No. 53468.

In specific embodiments of the invention, an enzymatically active human manganese superoxide dismutase analog is produced by E. coli strain A4255 cell containing the plasmid pMSE-4 and by E. coli strain A4255 cell containing the plasmid pMSΔ4RB4.

The suitable production medium for the bacterial cell can be any type of acceptable growth medium such as casein hydrolysate or LB (Luria Broth) medium, the latter being preferred. Suitable growth conditions will vary with the strain of E. coli and the plasmid it contains, for example E. coli A4255 containing plasmid pMSE-4 is induced at 42°C and maintained at that temperature from about 1 to about 5 hours. The suitable conditions of temperature, time, agitation and aeration for growing the inoculum and for growing the culture to a desired density before the production phase as well as for maintaining the culture in the production period may vary and are known to those of ordinary skill in the art.

The concentration of Mn^{++} ion in the medium that is necessary to produce enzymatically active

manganese superoxide dismutase will vary with the type of medium used.

In LB-type growth media Mn^{++} concentrations of 150 ppm to 750 ppm have been found effective. It is preferred that in all complex types of growth mediums the concentration of Mn^{++} in the medium is from about 50 to about 1500 ppm.

5 The specific ingredients of the suitable stock, culture, inoculating and production mediums may vary and are known to those of ordinary skill in the art.

This invention also concerns a method of recovering human manganese superoxide dismutase or analog or mutant thereof from bacterial cells which contain the same. The cells are first treated to recover a protein fraction containing proteins present in the cells including human manganese superoxide dismutase

10 or analog or mutant thereof and then the protein fraction is treated to recover human manganese superoxide dismutase or analog or mutant thereof.

In a preferred embodiment of the invention, the cells are first treated to separate soluble proteins from insoluble proteins and cell wall debris and the soluble proteins are then recovered. The soluble proteins so recovered are then treated to separate, e.g. precipitate, a fraction of the soluble proteins containing the

15 human manganese superoxide dismutase or analog or mutant thereof and fraction is recovered. The fraction is then treated to separately recover the human manganese superoxide dismutase or analog or mutant thereof.

The following is a description of a more preferred embodiment of the invention. First, the bacterial cells are isolated from the production medium and suspended in a suitable solution having a pH of about 7.0 or

20 8.0. The cells are then disrupted and centrifuged. The resulting supernatant is heated for a period of about 30 to 120 minutes at a temperature between approximately 55 to 65°C, preferably for 45-75 minutes at 58 to 62°C and more preferably one hour at 60°C, and then cooled to below 10°C, preferably to about 4°C. Any precipitate which may form during cooling is removed, e.g. by centrifugation and then the cooled supernatant is dialyzed against an appropriate buffer. Preferably the cooled supernatant is dialyzed by

25 ultrafiltration employing a filtration membrane smaller than 30K, most preferably 10K. Appropriate buffers include 2 mM potassium phosphate buffer having a pH of about 7.8. After or simultaneously with this dialysis the cooled supernatant may optionally be concentrated to an appropriate volume, e.g. 0.03 of the supernatant's original volume has been found to be convenient. The retentate is then eluted on an anion exchange chromatography column with an appropriate buffered solu tion, e.g., a solution at least 20 mM

30 potassium phosphate buffer having a pH of about 7.8. The fractions of eluent containing superoxide dismutase are collected, pooled and dialyzed against about 40 mM potassium acetate, pH 5.5. The dialyzed pooled fractions are then eluted through a cation exchange chromatography column having a linear gradient of about 40 to about 200 mM potassium acetate (KOAc) and a pH of 5.5. The peak fractions containing the superoxide dismutase are collected and pooled. Optionally the pooled peak fractions may then be dialyzed

35 against an appropriate solution, e.g. water or a buffer solution of about 10 mM potassium phosphate having a pH of about 7.8.

The invention also concerns purified, i.e. substantially free of other substances of human origin, human manganese superoxide dismutase or analogs or mutants thereof produced by the methods of this invention. In particular, it concerns a human manganese superoxide dismutase analog having at least two polypep-

40 tides, at least one of which polypeptides has the amino acid sequence shown in Figure 1, the N-terminus of which sequence is the lysine encoded by nucleotides 115-117 of Figure 1 and the COOH terminus of which sequence is the lysine encoded by nucleotides 706-708 of Figure 1 plus an additional methionine residue at the N-terminus (Met-hMnSOD). A preferred embodiment of this invention concerns purified Met-hMnSOD having a specific activity of 3500 units/mg.

45 The invention further concerns the ligation of human manganese superoxide dismutase gene fragments taken from various plasmids to yield a complete human manganese superoxide dismutase gene fragment which can be introduced into mammalian cells for the production of MnSOD. The various human manganese superoxide dismutase fragments isolated from the plasmids detail the nucleotide sequence of the genomic human manganese superoxide dismutase gene including coding and adjacent nucleotides as

50 well as a restriction map and organization of the gene.

The genomic gene commences at nucleotide 479 which is the first nucleotide of the ATG initiation codon and is underlined in Figure 6. The TAA termination codon for the genomic gene is at nucleotides 2022-2024. These numbers are arbitrary numbers merely to assist in identifying the nucleotide region.

A restriction map and organization of the genomic human manganese superoxide dismutase gene is

55 depicted in Figure 5.

As noted, portions of genomic manganese superoxide dismutase DNA are found in each of three different clones, pMSG11-1, pMSG4, and pMSG-1b also depicted in Figure 5 and the DNA from these clones was used to map the nucleotide sequence of Figure 6. Also shown in Figures 6 and 7 are the exon

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and intron regions of the human manganese superoxide dismutase gene.

The invention also relates to eucaryotic cells in which the DNA sequence encoding human manganese superoxide dismutase is expressed and to eucaryotic cells wherein the cell has been transformed with a plasmid comprising the DNA sequence encoding human manganese superoxide dismutase. This DNA
5 sequence may be present in the cell in single or in multiple copies. The DNA sequence encoding human manganese superoxide dismutase is derived from a fragment of human genomic DNA containing the human manganese superoxide dismutase gene.

In addition, the DNA sequence encoding human manganese superoxide dismutase is regulated by native regulatory elements of human genomic manganese superoxide dismutase. The complex DNA
10 sequence analog for genomic human manganese superoxdide dismutase polypeptide or an analog thereof including gene sequences coding for the native regulatory elements regulating gene expression is shown in the non-coding region of Figure 6 in the region prior to where the prepolypeptide is encoded.

The eucaryotic cell expressing the genomic sequence of human manganese superoxide dismutase is a mammalian cell. Examples of mammalian cells which may be used to express the genomic DNA sequence
15 are the human HeLa cell line and mouse L cells although it is to be understood that any mammalian cell line may be used.

The subject invention also relates to methods for producing human manganese superoxide dismutase comprising growing the eucaryotic cells described above in a suitable medium under suitable conditions permitting production of the human manganese superoxide dismutase, human manganese superoxide
20 dismutase analog, or human manganese superoxide dismutase mutant and permitting the recovery of the resulting human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant.

The subject application further relates to various uses of the human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants of
25 the invention.

The human superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants may be used, for example, to treat a subject suffering from a disorder associated with the generation of oxygen free radicals in quantities sufficient to cause undesirable symptoms by administering to the subject an amount of the human manganese superoxide dismutase,
30 analog or mutant to alleviate the undesirable symptoms. The compounds may be used to treat, for example, a subject with inflammation, and in particular, subjects having synovial inflammation. The inflammation may be induced by lipopolysaccharide endotoxin (LPS) or by any other inflammatory causing agent. The human manganese superoxide dismutase is preferably administered intraarticularly directly to the joint in amounts between about 1 to about 200 mg/joint applied in daily amounts. Other methods of administration may also
35 be used.

Human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants may further be used to treat arthritis and to treat the inflammation caused by arthritis. This arthritis may be induced, for example, by an adjuvant. The human manganese superoxide dismutase, the analogs or mutants are administered preferably subcutaneously in daily doses of
40 between about 1 and about 100 mg/kg body weight of the subject and more preferably in amounts of between about 3 and about 50 mg/kg body weight of the subject. The compounds may also be administered intravenously or by the other methods known to those skilled in the art.

The human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants may further be used to treat lung fibrosis, a symptom
45 often associated as a side effect of bleomycin. The human manganese superoxide dismutase, analogs or mutants are preferably administered subcutaneously in daily doses of between about 1 and about 100 mg/kg body weight of the subject and more preferably in amounts of between about 3 and about 50 mg/kg body weight of the subject. The compounds may also be administered intravenously or intratracheally.

The invention further relates to specific human manganese superoxide dismutase analogs in which one
50 of the human manganese superoxide dismutase polypeptides differs from naturally-occurring human manganese superoxide dismutase by the absence of one or more amino acids from the N-terminus.

The subject application is further concerned with pharmaceutical compositions comprising the human manganese superoxide dismutase, human manganese superoxide dismutase analogs or human manganese superoxide dismutase mutants in a suitable pharmaceutical carrier.
55 The compositions may be used for treatment of various disorders which include, but are not limited to, treatment of reperfusion injury following ischemia or organ transplants: treatment of inflammations: treatment of arthritis; treatment of bronchial pulmonary dysplasia. treatment of inflammation caused by Crohn's disease; and treatment of inflammation caused by collitis. The compositions alleviate the undesirable

symptoms associated with the generation of oxygen free radicals.

EXAMPLES

The Examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way. The Examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of genes encoding polypeptides into such vectors or the introduction of the resulting plasmids into hosts. The Examples also do not include detailed description for conventional methods employed for assaying the polypeptides produced by such host vector systems or determining the identity of such polypeptides by activity staining of isoelectric focusing (IEF) gels. Such methods are well-known to those or ordinary skill in the art and are described in numerous publications including by way of example the following:

T. Maniatis, E.F. Fritsch and J. Sombrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982).

J.M. McCord and I. Fridovich, J. Biol. Chem. 244: 6049-55 (1969).

C. Beauchamp and I. Fridovich, Anal. Biochem. 44: 276-87 (1971).

EXAMPLE 1

In order to identify manganese superoxide dismutase cDNA clones, mixed oligomer probes were synthesized according to the published amino acid sequence (18,19).

```
5'                                    3'

TTGCATAATTTGTGCCTTAATGTGTGGTTC
         T      G      T      G
         G             G
```

3'-probe - 32 mer sequence from $AA_{179}-AA_{189}$ (18)

```
5'                                    3'

TCTGTTACGTTTTCCCAGTTTATTACGTTCCA
   G  G                 G  G
```

The 5'-probe consisting of 30 nucleotides corresponds to amino acids 15 to 24 of mature MnSOD. The 3'-probe consisting of 32 nucleotides corresponds to amino acids 179 to 189 of mature manganese superoxide dismutase. The 5'-probe is a mixed probe consisting of 36 different sequences, as shown above. The 3'-probe is a mixed probe consisting of 16 different sequences as shown above. (When more than one nucleotide is shown at a given position, the DNA strand was synthesized with equimolar amounts of each of the shown nucleotides thus resulting in the mixed probe).

The 5'-probe was employed to screen 300,000 plaques of a T-cell cDNA library cloned into the λ gt-10 vector. Hybridization to phage plaque replicas immobilized on nitrocellulose filters was performed according to standard procedures (Maniatis et al. supra) except that the hybridization was performed at 50°C in 8×SSC for 16 hrs. The filters were then washed at 50°C with 5×SSC and 0.1% SDS. Three positive plaques were isolated and named Phi MS8, Phi MS1 and Phi MS1J.

EcoRI digests of DNA from Phi MS8 and Phi MS1 showed that they both have cDNA inserts approximately 800 bp long, which hybridize to both the 5' and 3' oligonucleotide probes. Phi MS1J carried only 450 bp cDNA insert which hybridized only to the 5' end probe.

The EcoRI inserts of the three phage clones were subcloned into the EcoRI site of pBR322 thus yielding pMS8-4, pMS1-4 and pMS1J, respectively. Restriction analysis and hybridization to the 5′ and 3′ oligonucleotide probes revealed similar patterns for both pMS8-4 and pMS1-4. The following restriction map showing the 5′ → 3′ orientation has been deduced for both plasmids.

5

```
5′
   R1       PvuII   PvuII   ScaI            BamI  StuI              R1
   ↓         ↓       ↓       ↓               ↓     ↓                ↓
  ─────┬───────────┬───────┬───────┬───────┬─────┬───────┬────────
       100       200     300     400     500   600     700      800
```

15
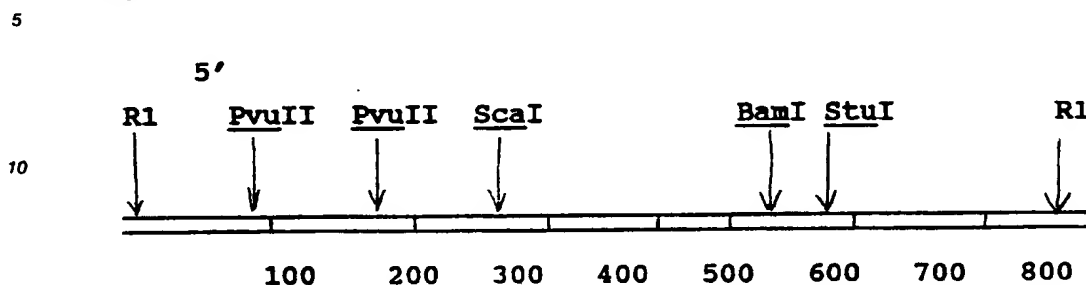
The sequence of the cDNA insert of pMS8-4 is shown in Figure 1. The predicted amino acid sequence differs from the published amino acid sequence (19) in that Glu appears instead of Gln in three (3) locations (AA 42, 88, 108) and an additional two amino acids, Gly and Trp appear between AA_{23-124}. Sequence analysis of pMS1-4 and pMS1J revealed that the three manganese superoxide dismutase clones were independently derived and confirmed these differences from the published amino acid sequence.

The sequence upstream of the N-terminal Lysine of mature manganese superoxide dismutase predicts a prepeptide sequence of 24 amino acids.

EXAMPLE 2

Construction of pMS8-4: Amp^R Human Manganese Superoxide Dismutase Expression Plasmid

The starting point for the construction of pMSE-4 is the plasmid pMS8-4 which was obtained as described in Example 1. Plasmid pMS8-4, containing human manganese superoxide dismutase cDNA on an EcoRI insert, was digested to completion with Ndel and Narl restriction enzymes. The large fragment was isolated and ligated with a synthetic oligomer as depicted in Figure 2. The resulting plasmid, pMS8-NN contains the coding region for the mature manganese superoxide dismutase, preceded by an ATG initiation codon. The above plasmid was digested with EcoRI, ends were filled in with Klenow fragment of Polymerase I and further cleaved with Ndel. The small fragment containing the manganese superoxide dismutase gene was inserted into pSODα13 which was treated with Ndel and Stul. pSODα13 may be obtained as described in pending, co-assigned U.S. Patent Application Serial No. 644,245, filed August 27, 1984 which is hereby incorporated by reference. This generated plasmid pMSE-4 containing the manganese superoxide dismutase coding region preceded by the cII ribosomal binding site and under the control of λ P$_L$ promoter. Plasmid pMSE-4 has been deposited with the American Type Culture Collection under ATCC Accession No. 53250. All methods utilized in the above processes are essentially the same as those described in Maniatis, supra.

EXAMPLE 3

Expression of the Recombinant Human Manganese Superoxide Dismutase

Plasmid pMSE-4 was introduced into Escherichia coli strain A4255 using known methods. Then the E. coli strain 4255, containing pMSE-4, were grown at 32°C in Luria Broth (LB) medium containing 100 μg/ml of ampicillin until the Optical Density (OD) at 600 nm was 0.7. Induction was performed at 42°C. Samples taken at various time intervals were electrophoresed separated on sodium dodecyl sulfate - polyacrylamide gels electrophoresis (SDS-PAGE). The gels showed increases in human manganese superoxide dismutase levels up to 120 minutes post-induction, at which stage the recombinant manganese superoxide dismutase protein comprised 27% of total cellular proteins as determined by scanning of Coomassie-blue stained gel. Sonication of samples for 90 sec. in a W-375 sonicator and partitioning of proteins to soluble (s) and non-soluble (p) fractions by centrifugation at 10,000 μg for 5 min. revealed that most of the recombinant manganese superoxide dismutase produced was non-soluble. The induced soluble protein fraction con-

tained only slightly more SOD activity than the uninduced counterpart, as assayed by standard methods. See McCord et al., _supra_. Apparently a portion of the manganese superoxide dismutase found in the soluble fraction is inactive. This suggested that most of the human manganese superoxide dismutase produced under the conditions described in this Example is, in effect, inactive.

EXAMPLE 4

Effect of Mn^{++} in Growth Media on Manganese Superoxide Dismutase Solubility and Activity

The addition of Mn^{++} in increasing concentrations up to 450 ppm to the growth media of E. coli A4255, containing pMSE-4, prior to a 2 hr. induction at 42° C had no adverse effect on the overall yield of human manganese superoxide dismutase. Analysis of sonicated protein fractions soluble (s) and non-soluble (p) on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), showed increased solubilization of the recombinant protein with increased Mn^{++} concentrations (Table 1). An assay of superoxide dismutase activity (see McCord et al., supra) suggests a correlation between increased Mn^{++} concentrations in the growth media and increased solubility of the manganese superoxide dismutase with an apparent optimum at 150 ppm Mn^{++} concentration in the media (Figure 3). Furthermore increased Mn^{++} concentrations activated previously inactive soluble enzyme. Soluble protein fractions of induced cultures grown at these Mn^{++} levels show up to 60-fold increase in SOD activity over soluble protein fractions of non-induced cultures grown at these Mn^{++} levels. Activity staining of isoelectric focusing (IEF) gels (see Beauchamp et al, _supra_.) revealed that multi forms of the recombinant manganese superoxide dismutase were identical to those of native human liver MnSOD.

Results for human manganese superoxide dismutase production by E. coli A1645 containing pMSE-4 were similar to those described above.

TABLE 1

Mn^{++} (ppm)	Percent Soluble human Mn SOD of Total human MnSOD Induced	Percent Soluble human Mn SOD of Soluble Bacterial Proteins	Specific Activity units/mg Soluble Proteins
0	30.6	7.2	30
50	72.7	15.4	241
100	78.0	16.9	356
150	82.9	18.8	606
200	82.0	20.8	338
250	79.2	20.4	380
300	80.8	20.3	381
450	89.2	22.4	323

EXAMPLE 5

Construction of pMSΔRB4: Tet^R Human Manganese Superoxide Dismutase Expression Plasmid

5   Tet^{R}expression vector, pΔRB, was generated from pSODβ,T-11 by complete digestion with EcoRI followed by partial cleavage with BamHI restriction enzymes. pSODβ,T-11 has been deposited with the American Type Culture Collection under Accession No. 53468. The digested plasmid was ligated with synthetic oligomer

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$$5' - \text{AATTCCCGGGTCTAGATCT} - 3'$$
$$3' - \qquad \text{GGGCCCAGATCTAGACTAG} - 5'$$

15   resulting in pΔRB containing the λ P$_L$ promoter.

The EcoRI fragment of manganese superoxide dismutase expression plasmid pMSE-4, containing cII ribosomal binding site and the complete coding sequence for the mature enzyme, was inserted into the unique EcoRI site of pΔRB. The resulting plasmid, pMSΔRB4, contains the manganese superoxide dismutase gene under control of λ P$_L$ and cII RBS and confers resistance to tetracycline (Figure 4).

20

EXAMPLE 6

Expression of Human Manganese Superoxide Dismutase from pMSΔRB4

25   Plasmid pMSΔRB4 was introduced into Escherichia coli strain A4255, using known methods. Cultures were grown at 32°C in Luria Broth (LB) containing various concentrations of Mn^{++}, until the Optical Density (OD) at 600 nm reached 6.7. Induction was performed at 42°C. Samples taken at various time intervals were electrophoresed on SDS-PAGE. hMnSOD level increased with induction time up to 120 minutes, at which stage it comprised about 15% of total cellular proteins as determined by scanning of Coomassie Blue

30   stained gel.

The induced manganese superoxide dismutase was soluble, regardless of Mn^{++} concentration in growth media. This is in contrast with observations for the Amp^{R}plasmid pMSE-4. (See Example 4.) However, maximum SOD activity and expression level were dependent on Mn^{++} supplementation (Table 2).

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TABLE 2

Manganese Superoxide Dismutase Expression in E.Coli A4255 (pMSΔ RB4)

ppm Mn^{++}	Percent Soluble hMnSOD of Soluble Bacterial Proteins	Specific Activity Units/mg Soluble Proteins	
	42°	32°	42°
0	10.9	8.0	23
50	19.8	8.0	227
100	16.0	8.0	241
200	17.0	10.0	278
300	16.0	9.3	238

EXAMPLE 7

Purification of Enzymatically Active Recombinant Human Manganese Superoxide Dismutase Analog

E. coli strain A4255 harboring plasmid pMSΔRB4 was fermented in LB supplemented with 750 ppm Mn^{++}, at 32°C to an A600 of 17.0. Induction of human manganese superoxide dismutase expression was effected by a temperature shift to 42°C for 2 hours at which stage the culture reached A600 of 43.0. Cells were harvested by centrifugation and resuspended in 0.2 original volume in 50 mM potassium phosphate buffer, pH 7.8 containing 250 mM NaCl. Bacteria were disrupted by a double passage through Dynomill®, centrifuged and cell debris were discarded. The supernatant was heated for 1 hour at 60°C, cooled to 4°C and the cleared supernatant was concentrated to 0.03 original volume and dialyzed against 2 mM potassium phosphate buffer, pH 7.8, on a Pellicon® ultra filtration unit equipped with a 10K membrane. The crude enzyme preparation was loaded onto a DE52® column, washed thoroughly with 2 mM potassium phosphate buffer, pH 7.8 and eluted with 20 mM potassium phosphate buffer, pH 7.8. Pooled fractions containing the enzyme were dialyzed against 40 mM potassium acetate, pH 5.5, loaded onto a CM52® column and eluted with a linear gradient of 40 - 200 mM potassium acetate, pH 5.5. Peak fractions containing human manganese superoxide dismutase were pooled, dialyzed against H_2O, adjusted to 10 mM potassium phosphate buffer, pH 7.8 and frozen at -20°C.

Recombinant human manganese superoxide dismutase analog obtained was more than 99% pure, with a specific activity of about 3500 units/mg. The overall yield of the purification procedure was about 30% (Table 3).

Sequencing of the purified enzyme shows the presence of an additional methionine at the N-terminal amino acid as compared with the known human manganese superoxide dismutase (19).

Analysis for metal content by atomic absorption revealed about 0.77 atoms Mn per enzyme subunit. This is in accordance with published data (23).

TABLE 3

Purification of Recombinant Human* Mn-SOD

Step	Total Proteins gm	Yield gmSOD	%	Specific Activity units/mg
Dynomill* supernatant	100.0	11.9	100.0	417
60°C supernatant	24.0	8.2	68.9	1197
Pellicon* retentate	20.0	7.5	63.0	1350
DE52* eluate	7.3	5.7	48.0	2732
CM52* eluate	4.2	4.2	35.3	3500

*Values for enzyme purified from 15 L fermentation.

EXAMPLE 8

Isolation and Structure of Human Manganese Superoxide Dismutase Gene

Human placenta DNA digested with HindIII and BamHI was fractionated according to its size, hybridized with an manganese superoxide dismutase cDNA probe and the positive enriched fractions were cloned in pBR322. Three distinct clones were identified according to their restriction and hybridization patterns: pMSG11-1, overlapping pMSG4, both comprising the 5' end of manganese superoxide dismutase gene and followed by the consecutive clone pMSG-1b which contains the 3' end of the gene. Plasmid pMSG11-1 has been deposited in the ATCC under Accession No. 67363; plasmid pMSG4 has been deposited in the ATCC under Accession No. 67364; and plasmid pMSG-1b has been deposited in the ATCC under Accession No. 67365. Figure 5 shows the restriction map and organization of the manganese superoxide dismutase gene. The nucleotide sequence of the gene is shown in Figure 6. The manganese superoxide dismutase gene spans a region of about 15 Kb and contains six exons. The first intron interrupts the region coding for the leader peptide while the last intron appears in the 3' untranslated region downstream to the TAA termination codon.

The sequences of the donor and acceptor splice junctions at the exon-intron boundries are summarized in Figure 7 and compared with the consensus sequence. It should be pointed out that the first intron contains either an unusual donor sequence; GG instead of the highly conserved GT (as depicted in Figure 6), or an unusual acceptor sequence; GG instead of AG (if one moves the exon by one nucleotide). All other four introns are bound by the conserved GT ... AG nucleotides.

The promoter region lacks TATA and/or CAT boxes. However, it is highly rich in GC and contains eight repeats of the consensus hexanucleotide core for binding transcription factor Spl (GGGCGG). Moreover, it includes a series of direct repeats and possible stem-loop structures. The polyadenylation signal AATAAA appears 85 nucleotides downstream from the last exon (according to the known cDNA sequence). The sequence of the promoter region and the sequence of the coding regions and adjacent nucleotides was determined.

The manganese superoxide dismutase gene regions from plasmids pMSG11-1, pMSG4 and pMSG-1b can be conveniently isolated from the plasmids and ligated to one another to form the entire manganese superoxide dismutase gene. For example, the approximately 6 KB HindIII-partial BamHI fragment from

pMSG11-1 can be ligated to the entire BamHI insert in plasmid pMSG4 followed by ligation to the entire BamHI insert from pMSG-1b. The result of this ligation would be a DNA fragment encoding the human manganese superoxide dismutase gene. This DNA fragment could then be introduced into mammalian cells through known methods either directly or after ligation to a cloning vehicle such as a plasmid or virus. The transformed cell line could then be used for production of manganese superoxide dismutase polypeptide, analog or mutant thereof, by culturing in a suitable medium under suitable conditions. The polypeptide so produced could then be recovered by methods similar to those set forth in Example 7. The polypeptide so recovered could then be used formulated and used therapeutically, for example, for treatment of ischemia or inflammation.

EXAMPLE 9

Transcription of Manganese Superoxide Dismutase in Human Cells

The human manganese superoxide dismutase cDNA from plasmid pMS-84 (Figure 2) was hybridized to polyA$^+$ RNA from human cell lines, human placenta, mouse WEHI-3 cells and bovine liver. Two species of human mRNA for manganese superoxide dismutase were identified, a major transcript of human mRNA encoding manganese superoxide dismutase of about 1000 nucleotides (nt) long and a minor transcript of about 4000 nt in length. The mouse mRNA for manganese superoxide dismutase is similar in size to the human major transcript, whereas mRNA for bovine manganese superoxide dismutase is about 300 nt longer. The long human trancript (4000 nucleotides) hybridizes to the fifth intron of the human manganese superoxide dismutase gene, downstream from the exon coding for the carboxy terminus of the enzyme. This partially spliced transcript is non-tissue specific.

The proportion of both CuZn and manganese superoxide dismutase mRNAs in various cell lines was in the order of 10^{-3}%, as determined by hybridization of the SOD cDNA probes to dot blots of serially diluted polyA$^+$ RNA (Table IV). The manganese superoxide dismutase message was most abundant in Hepatoma cells (2.5×10^{-3}%) and CuZn SOD transcripts were most abundant in the T-lymphocyte line (4×10^{-3}%).

TABLE IV

Transcription Levels of Manganese Superoxide Dismutase and CuZn SOD in Human Cell Lines

% of polyA$^+$ RNA

Cell Line	MnSOD	CuZnSOD
1. PEER T-cell	0.6×10^{-3}	4.0×10^{-3}
2. 5637 Bladder Carcinoma	0.8×10^{-3}	1.6×10^{-3}
3. Alexander Hepatoma	2.5×10^{-3}	2.0×10^{-3}

EXAMPLE 10

Pharmacokinetic and Anti-Inflammatory Properties of Recombinant Human Manganese Superoxide Dis-mutase

INTRODUCTION

5

The anti-inflammatory activity of CuZn SOD has been demonstrated in various biological models. The pharmacokinetics of CuZn SOD after administration by various routes have also been examined, and it has been found to have a relatively short half-life (approximately 7 minutes when injected intravenously). By contrast, very little is known about the pharmacokinetics and biological activity of MnSOD. There is only one
10 report on the comparison of the pharmokinetic and anti-inflammatory properties of the CuZn and the Mn containing enzymes (Baret et al., 1984). Baret et al. claim that the half-life of manganese superoxide dismutase injected intravenously is extremely long (6.45 hours). On the other hand, manganese superoxide dismutase was shown to be ineffective against carrageenan-induced paw inflammation in rats, while the CuZn SOD was effective in reducing inflammation. In the study described herein, we have compared the
15 pharmacokinetic properties of recombinant human manganese superoxide dismutase with that of a recom-binant human CuZn SOD analog when given subcutaneously. Concurrently, we have compared the anti-inflammatory activities of these two enzymes in the carrageenan paw edema model. Unexpectedly, it was found that manganese superoxide dismutase was efficacious in reducing inflammation in the rat model system.

20

PHARMACOKINETIC STUDIES

Rats were injected subcutaneously with 50 mg/kg body weight of either recombinant human CuZn SOD
25 analog or recombinant human MnSOD. Blood samples were drawn 0.5, 2, 4, 8, 24, 30 and 48 hours after injection and superoxide dismutase activity in the samples was determined by an enzymatic assay (Fridovich).

Figure 8 summarizes the results. As shown, CuZn SOD analog values reached a maxmimum of about 10 μg/ml after 2 hours and stayed at about that level for additional 6 hours, but dropped to pre-injection
30 levels after 24 hours. By contrast, manganese superoxide dismutase levels gradually increased to reach a maximal level of about 70 μg/ml by 8 hours, and stayed at about this level for at least 30 hours. By 48 hours, the enzyme activity in serum dropped to about 20 μg/ml, a value that was still well above the pre-injection levels.

35

ANTI-INFLAMMATORY ACTIVITY

The rat model of carrageenan-induced paw edema was used to assay the anti-inflammatory activity of CuZn SOD analog and MnSOD. In this model, Wistar-derived male rats (130-150g b.w.) were given a sub-
40 plantar injection of 0.1 ml of 0.1% w/v carrageenan into the left hind paw. The paw volume was measured by an Hg-displacement volumeter (a modification of a Ugo-Basile® volumeter, Comerio, Italy) before and at hourly intervals after paw injection. Animals were divided into 4 groups (8 rats/group). One group received a subcutaneous injection of 50 mg/kg of manganese superoxide dismutase 24 hours before carrageenan administration (-24 h Mn; cf. Figure 9). The second group was injected with Cu/Zn SOD analog (50 mg/kg)
45 24 hours before the carrageenan challenge (-24 h Cu), while the third group was injected with 60 mg/Kg of CuZn SOD analog only 2 hours before the challenge (-2h Cu). The fourth group did not receive any pretreatment and served as a control.

The results are shown in Figure 9. As seen, the administration of CuZn SOD analog 2 hours before the induction of inflammation resulted in a 50% reduction of the swelling response. In contrast, pretreatment
50 with the CuZn enzyme 24 hours before challenge was without effect. However, a 24-hour pretreatment with manganese superoxide dismutase resulted in an anti-inflammatory response which was similar to the effect of the 2 hours pretreatment with CuZn SOD analog.

55

CONCLUSIONS

It has been demonstrated herein that the rate of disappearance of recombinant human manganese superoxide dismutase in the rat is much lower than that of the recombinant CuZn SOD analog. This is in
5   agreement with the previous report of Baret et al. (1984) concerning the natural human manganese superoxide dismutase. However, the manganese-containing enzyme has been shown herein to be active in vivo as an anti-inflammatory agent - an activity that is attributed to its superoxide dismutase ability. This finding is surprising in view of the report of Baret et al. (1984), which claimed that manganese superoxide dismutase was not active in a similar system. The finding that manganese superoxide dismutase remains
10   efficacious as an antiinflammatory drug even 24 hours after administration indicates that it may be used as a long-acting therapeutic agent.

EXAMPLE 11

15

Biological Model of Recombinant Manganese Superoxide Dismutase and its Role as an Anti-Inflammatory Agent: Pharmacokinetics of Intravenously Administered MnSOD

Male rats (100-110 gram body weight, group size of 7 rats) were injected intravenously with human
20   recombinant manganese superoxide dismutase prepared in saline solution (saline = 0.15 M NaCl). Dosage of manganese superoxide dismutase was according to rat body weight (b.w.) and in this study, 25 mg/kg b.w. was the dosage of choice.

Intravenous (i.v.) administration was compared pharmacokinetically with intraperitoneal (i.p.) and sub-cutaneous (s.c.) administrations using the same dose (25 mg/kg b.w.) of recombinant manganese superox-
25   ide dismutase in saline and the same number of rats per group (n = 7).

After administration of manganese superoxide dismutase in the above three groups, blood samples were drawn at 0.03, 0.5, 2, 4, 24, 30 and 48 hours post-injection and were analyzed both enzymatically and by RIA. The enzyme assay was exactly as described before in Example 10 (using method of Fridovich). The RIA was carried out using standard procedures with anti-manganese superoxide dismutase antibodies
30   (prepared by us) and protein A (from Staphylococcus aureaus).

The results are depicted in Figure 10 and may be summarized as follows:

(1) Intravenously administered (i.v., curve (1)) manganese superoxide dismutase has a very high initial serum level, as expected, of 430 μg/ml. This level drops sharply in the first two hours following injection (to 240 μg/ml). This sharp drop is due to the re-distribution (via uptake mechanisms) of
35   manganese superoxide dismutase throughout the various tissues of the body. After the initial two hours, the rate of clearance of manganese superoxide dismutase has first-order kinetics and a half life, $t1/2$, of 6.2 hours.

(2) Intraperitoneally administed (i.p., curve (2)) manganese superoxide dismutase showed an initial (first 2 hours) sharp increase in serum manganese superoxide dismutase levels, consistent with rapid
40   uptake via the peritoneal membranes, (from 0 μg/ml to 115 μg/ml). The subsequent increase in the next two hours is slow, reaching peak serum levels of 120 μg/ml after 4 hours. At this point serum manganese superoxide dismutase levels began to decline with a first-order clearance rate, and a t 1/2 of 5.5 hours.

(3) Subcutaneously administered manganese superoxide dismutase shows a low rate of entry into the serum, reaching low levels of only 27 μg/ml after 4 hours. At this point serum manganese superoxide
45   dismutase levels decline with a first-order clearance rate and t 1/2 of 7.3 hours.

It should also be noted, especially in the case of intravenously administered recombinant manganese superoxide dismutase, that significant serum levels are still present 48 hours after injection (10 μg/ml).

Thus, we conclude that the most effective way of administering manganese superoxide dismutase is intravenously. This gives very high initial serum levels which remain as significantly high serum levels (160
50   μg/ml) in the first 4 hours after injection. This is much more effective than either intraperitoneally or subcutaneously-administered manganese superoxide dismutase using same dosages in injection.

Finally, in all cases, the t 1/2 of serum manganese superoxide dismutase is markedly greater than that described for CuZn SOD (about 7 - 30 minutes), thus making manganese superoxide dismutase a potentially more effective therapeutic agent than CuZn SOD.

55   Human manganese superoxide dismutase was reported to have a much longer half-life after intravenous administration to rats than the phylogenetically unrelated enzyme CuZn SOD ($t1/2$ = 6.45 h vs. 6 min, respectively; Baret, Jadot and Michelson, Biochem. Pharmacol. 33: 2755 (1984).) It was further claimed that, unlike CuZn SOD, manganese superoxide dismutase is pharmacologically inactive when tested in inflamma-

tion models in vivo. As described above, we have cloned and expressed human manganese superoxide dismutase in E. coli. The purified recombinant protein is different from the natural enzyme by the presence of an additional methionine at the amino terminus. However, it exhibits specific enzymatic activity indistinguishable from that of natural mitochrondrial manganese superoxide dismutase purified from human liver.

Pharmacokinetic analysis of recombinant human manganese superoxide dismutase (r-hMnSOD) given intravenously to rats revealed a half-life of 6.2 h, which is consistent with the published value for the natural enzyme. The potential anti-inflammatory activity of r-hMnSOD was tested in the model of carrageenan-induced edema in the rat paw. Recombinant human CuZn SOD (r-hCuZnSOD) was used for comparison. The two enzymes were administered according to two experimental regimens: (1) the enzymes were injected subcutaneously (50 mg/kg B.W.) either 2 h or 24 h before the carrageenan challenge; (2) the enzymes were injected intravenously at 8 or 20 mg/kg B.W. 10 min. prior to the carrageenan challenge. The results are illustrated in Table 5 below:

TABLE 5

Route of Injection	Time Before Carrageenan	Dose (Mg/Kg B.W.)	Inhibition of Paw Swelling	
			r-hMnSOD	r-hCuZnSOD
Subcutaneous	2 h	50	50%	50%
Subcutaneous	24 h	50	40%	0
Intravenous	10 min	8	50%	0
Intravenous	10 min	20	80%	10%

Collectively, the results demonstrate that r-hMnSOD is an effective ani-inflammatory agent. Furthermore, its extended half-life in the blood, relative to CuZn SOD, makes r-hMnSOD more suitable for treatment of chronic inflammatory diseases, such as arthritis, colitis and radiation damage.

EXAMPLE 12

Biological Model of Recombinant Manganese Superoxide Dismutase and Its Role as an Anti-Inflammatory Agent: Effect of Intra-Articular Administration of Manganese Superoxide Dismutase on Synovial Inflammation Induced by Bacterial Lipopolysaccharide Endotoxin (LPS)

As a model for synovial inflammation and the ability of manganese superoxide dismutase to inhibit such inflammation, the knee-joint (synovial) inflammation model in rats was utilized. (More details used herein as reference, are described by Ginsburg et al., in "Bayer-Symposium VI: Experimental Models of Chronic Inflammatory Diseases" 256-299 (1977), Springer Verlag).

In this model, inflammation is induced by intraarticular injection of bacterial lipopolysaccharide endotoxin (LPS) (0.1 mg/rat). The degree of inflammation is reflected by the swelling of the synovial tissue, and is measured as the increase in weight 8-16 hours postadministration. Figure 11 shows a comparison between human CuZn SOD and manganese superoxide dismutase administered together with the LPS. To test the anti-inflammatory effects of manganese superoxide dismutase and CuZn SOD in this model, it is best to coadminister the LPS toxin and the manganese superoxide dismutase or CuZn SOD. Dosages that were used are as follows: 0.6 mg/knee manganese superoxide dismutase and both 1 mg/knee and 5 mg/knee for CuZn SOD. As shown, manganese superoxide dismutase was as effective as CuZn SOD in the inhibition of LPS-induced synovial inflammation. Both doses of CuZn SOD (1 mg/knee, 5 mg/knee) and the

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dose of manganese superoxide dismutase (0.6 mg/knee) were effective in reducing, significantly, the amount of LPS-induced knee-joint inflammation. In fact, the level of inflammation resulting from co-injection of SOD (CuZn SOD or MnSOD) and LPS is no greater than the sham-injection with saline only (negative control). This therefore, clearly illustrates the anti-inflammatory effect of SOD (MnSOD and CuZn SOD). The inhibition of inflammation was judged to be significant on the basis of Student t-test statistical analysis at 3 levels of probabilistic stringency; 5%, 1% and 0.1% levels (p 0.05; - 0.01, and p 0.001, respectively). Thus, the higher CuZn SOD dose (5 mg/knee) was most significant in its effect; the manganese superoxide dismutase dose (0.6 mg/knee) and lower CuZn SOD dose (1 mg/knee) were highly significant in inflammation-inhibition.

EXAMPLE 13

Biological Model of Recombinant Manganese Superoxide Dismutase and Its Role as an Anti-Inflammatory Agent: Effect of Manganese Superoxide Dismutase in Adjuvant-Induced Arthritis

The induction of joint inflammation in rats by administration of Freund's adjuvant is considered to be the model of choice for experimental rheumatoid arthritis (more details, used herein as reference are described in Newbould, Brit. J. Pharmacol. 21 (1963) 127-). An injection of the adjuvant (50 μl of Freunds' adjuvant with 10 mg/ml of killed Mycobacterium Tuberculosis) into the foot pad results in an initial swelling of the paw, reaching a plateau after 3 days, followed after 14 days by a second increase in paw and joint swelling, which persists for another 7-10 days. The second period is regarded as the phase of immunologically-induced chronic arthritis. To examine the efficacy of SOD as an anti-arthritic drug, CuZn SOD and manganese superoxide dismutase were given subcutaneously (in doses expressed as mg per kg body weight) to adjuvant-treated rats during days 14-21 after adjuvant administration. The enzymes were given either daily (both 12.5 mg/kg and 25 mg/kg doses) or on alternating days (a 50 mg/kg dose). The results shown in Figure 12 indicate that manganese superoxide dismutase is more effective as the treatment given daily, while the CuZn SOD given on alternating days appeared to be ineffective. The levels of effectiveness of treatment were determined by statistical analysis using the Student t-test at 3 levels of stringency (described in Example 12). These results clearly demonstrate the efficacy of manganese superoxide dismutase as an anti-arthritic drug.

EXAMPLE 14

Biological Model of Recombinant Manganese Superoxide Dismutase and Its Role as an Anti-Inflammatory Agent: Effect of Manganese Superoxide Dismutase on Bleomycin-induced Lung Fibrosis

Bleomycin (BLM) is an antineoplastic agent widely used against various types of carcinomas and lymphomas (more details, used herein as reference, are described in Counts et al., J. Pharmacol. Exp. Ther. 219: 675-678 (1981). A major problem associated with the use of bleomycin is the induction of lung fibrosis resulting from high doses of the agent, thus limiting its therapeutic usefulness. (See, for example, Crooks & Bradner, J. Med., 7: 333 (1976)). This side-effect of bleomycin is believed to be mediated by the generation of oxygen radicals in the lung tissue. (See, for example, Frank, Trends Pharmacol. Sci. 4: 124 (1983)). To examine the possible use of manganese superoxide dismutase for the protection of the lung from bleomycin-induced fibrosis, we have used the rat as a biological model. (See, for example, Kelley et al., J. Lab. Clin. Med. 96: 254 (1980)). In this model, 15 rats (100-120 g b.w.) were treated with 1.5 mg (= 1.5 units) of bleomycin given intra-tracheally. A second group of rats (15 rats), the negative control, received saline only, while a third group (15 rats) was given bleomycin as in group 1 and then treated for 7 days with daily subcutaneous injections of manganese superoxide dismutase (50 mg/kg b.w. dose). Table 6 represents the 3 week mortality data following bleomycin administration. As seen in the table, the daily treatment with manganese superoxide dismutase was significantly protective (87% survival versus 40%; statis tical significance of p>0.05 by Student t-test). The lungs of the surviving rats were excised and the collagen content (expressed as hydroxy-proline content) was determined, (using standard procedures according to Woessner in "The Methodology of Connective Tissue Re-search", Hall, Ed. Oxford (1976) Joynson-Bruvvers Ltd. pp. 227-233 and Woessner. Arch. Biochem. Biophys. 93: 440-447 (1961). FIGURE 13 shows the lung weights (normalized for 100 g body weight) and hydroxyproline content of the treated animals. As shown in Figure 13, the MnSOD treatment partially inhibited the increase in total lung weight and effectively

protected the lungs against an increase in collagen (reflected by level of hydroxyproline).

As seen in Figures 13 and 14, OH-proline levels in the r-human manganese superoxide dismutase treated group were elevated to only 25% of the BLM group, indicating that the enzyme effectively inhibited fibrosis.

5 In two additional experiments, we compared the effects of frequency as well as different routes of administration using both r-human manganese superoxide dismutase and r-human CuZn SOD. In the first experiment, the enzymes were given subcutaneously (50 mg/kg) either once every other day (Days 1, 3, 5) or by a single injection on Day 1. As seen in Figure 15, r-human manganese superoxide dismutase was partially protective when given once (approx. 34% reduction of BLM result) but was more effective when
10 administered every other day or daily (Figures 14 and 15) for a week (nearly 75% reduction of BLM insult). Similarly, a single dose of r-human CuZn SOD showed no protection, but when given every other day was equally as protective as a single injection of r-human manganese superoxide dismutase.

The same experiment was repeated using an intraperitoneal route of administration rather than subcutaneous. The results, depicted in Figure 16, again show that the preferred combination is r-human
15 manganese superoxide dismutase given three times a week.

Collectively, our results confirm the hypothesis that BLM in lung fibrosis is oxygen free radical mediated, and that such fibrosis could be inhibited by either r-human manganese superoxide dismutase or r-human CuZn SOD. The fact that r-hMnSOD was more effective than r-CuZnSOD in this process is most probably due to its longer half-life in circulation following intravenous injection (t 1/2 i.v. for r-hMnSOD and
20 CuZn SOD is about 6 hours versus 6 minutes, respectively, in rats). Figure 10 represents the results of our pharmacokinetic study of r-human manganese superoxide dismutase in rats (25 mg/kg), using 3 routes of administration.

The first-order kinetic decline from peak levels showed half-lives of 6.2, 5.5 and 7.3 hours for intravenous, intraperitoneal and subcutaneous route respectively. The much shorter half-life of r-human
25 CuZn SOD (Figure 8) requires more frequent administration and possibly higher doses as compared to r-human manganese superoxide dismutase, in order to attain equivalent pharmacological levels and hence similar protective effects. Further experiments assessing dose-response are in progress.

We conclude that the recombinant SOD enzymes, particularly r-human manganese superoxide dismutase, might be a useful adjunct to bleomycin (and possibly its derivatives, such as tallysomycin) therapy.
30

CONCLUSION

It has been demonstrated herein that recombinant manganese superoxide dismutase is an effective
35 anti-inflammatory agent. This was shown by its therapeutic effects in three biological models of inflammation. In all three models, the recombinant manganese superoxide dismutase had a highly significant effect in inhibiting inflammation and was at least as effective (in most cases more effective) than CuZn SOD which was used for comparative purposes. In addition, the fact that manganese superoxide dismutase remains in the serum for long periods, as shown by the pharmacokinetic studies, indicates that it may be used as a
40 long-acting therapeutic agent.

TABLE 6

Effect of MnSOD on Bleomycin-induced Death After 3 Weeks

Treatment	No. of Surviving Rats
Bleomycin(*)	6/15 (40%)
Bleomycin(*) + MnSOD(**)	13/15 (87%) (p<0.05: Student t-test)
Saline (0.15 M NaCl) only	15/15 (100%)

* 1.5 mg (1.5 units) Bleomycin was given intra-tracheally per rat.

** 50 mg/kg MnSOD was given daily per rat (by subcutaneous injection) for 7 days on the first week after bleomycin administration.

EXAMPLE 15

Preparation of Human Manganese Superoxide Dismutase Analogs

Human manganese superoxide dismutase was produced essentially as described in Example 7. The manganese superoxide dismutase has the following amino terminal sequence.

Met-Lys-His-Ser-Leu-Pro---

As described below, the manganese superoxide dismutase (MnSOD) was treated with Aeromonas aminopeptidase. Aeromonas aminopeptidase was prepared from the extracellular filtrate of Aeromonas proteolytica obtained from the American Type Culture Collection (ATCC Accession No. 15338), essentially according to J.M. Prescott and S.H. Wilkes, Methods Enzymol. 46: 530-543 (1976). The purification procedure included the following steps: sedimentation and filtration of bacteria, ammonium sulfate precipitation of the filtrate (367 g per liter), acetone fractionation (43.7% to 70% acetone), heat treatment at 70°C (for 8 hours) to destroy endopeptidase activity, gel filtration on Sephadex G-75® and ion-exchange chromatography on DEAE-Sephadex A-50®. In all experiments 10 mM Tris-Hcl buffer, pH 8.0 replaced 10 mM tricine buffer, pH 8.0 employed by Prescott and Wilkes. Pre-equilibration and elution from the Sephadex G-75® column were performed using a solution containing 5 micromolar (μM) ZnCl$_2$ rather than 50 micromolar (μM) ZnCl$_2$ employed in the original procedure (Prescott and Wilkes, supra). Purification on the DEAE-Sephadex A-50® column was performed by preequilibration of the column with 0.1 M NaCl in 10 mM Tris-Hcl, pH 8.0 containing 5 micromolar (μM) ZnCl$_2$, application of the sample and gradient elution

with 0.6 M NaCl in the same buffer (containing 5 micromolar (μM) ZnCl$_2$). When the salt concentration had reached about 0.5M the column was eluted with 0.7M NaCl in the same buffer. The major peak which eluted from the column was collected and dialyzed against 10 mM Tris-Hcl, 0.1M NaCl, pH 8.0 containing 5 micromolar (μM) ZnCl$_2$ and then kept frozen at -20°C.

5   Prior to reaction with the human manganese superoxide dismutase (hMnSOD), the analog or mutant (of hMnSOD), the amino peptidase enzyme solution was incubated at 70° for 2 hours to inactivate any possible traces of endopeptidase activity that might have been retained in the preparation and reactivated after prolonged storage.

    Amino acid analysis was performed using the method of precolumn derivatization with FMOC (9-
10 fluoroenylmethyloxycarbonyl chloride), (method according to Josefsson and Einarsson, see, for example, Einarsson et al., J. Chromatography 282: 609-618 (1983) and Einarsson, J. Chromatography 348: 213-220 (1985)). The amino acid derivatives were separated on a reverse phase Lichrocart Supersphere® (C18) column (Merck®) in a HPLC (high pressure liquid chromatography) system (Hewlett Packard® 1090 HPLC system, operated according to the manufacturer's specification).

    Depending on the pH of the solution during treatment, and depending on the duration of the treatment,
15 one or more amino acids were removed from the N-terminal of MnSOD. The pH was varied from pH 4.5 - pH 10 and the duration of treatment was extended to as long as 24 hours. The experiment below describes the currently preferred conditions for amino acid removal from the N-terminal of MnSOD.

    Met-MnSOD, 30 mg/ml, produced as described in Example 3, was dialyzed overnight in 5 mM
20 potassium acetate (KOAc) pH 4.5.

    250 microliters of Met-MnSOD solution, pH 4.5 (final concentration 6 mg/ml), 700 microliters of 5 mM KOAc buffer pH 4.5 and 50 microliters of aminopeptidase solution (1 mg/ml) were mixed and incubated at 37°C. Two aliquots of 108 microliters were taken after 0 h, 5 h and 24 h.

    To the first aliquot, 12 microliters of 15% sulfosalicylic acid solution in water were added, mixed and
25 incubated at 37°C for 15 min., and then centrifuged in an Eppendorf® bench centrifuge. 100 microliter sample was removed for direct amino acid analysis (without acid hydrolysis). The second aliquot was removed for N-terminal sequence analysis.

    Control experiments were run in parallel by incubating Met-MnSOD in the absence of aminopeptidase as described in the above procedure. Two aliquots of 108 microliters were removed at each time point, and
30 were treated and analyzed as described for the actual samples.

    After 24 hours of incubation at 37°C, the following molecules were obtained as illustrated in Table 7:

TABLE 7

Percent of Total Molecules	N-Terminal Sequence of SOD Analog
50%	Met-Lys-His-Ser-Leu-Pro
25%	Lys-His-Ser-Leu-Pro
12%	His-Ser-Leu-Pro
Less than 10%	Ser-Leu-Pro
Less than 10%	Leu-Pro

    The different analogs can be used for therapeutic purposes as described in Examples 10-14.

    If desired the different analogs can be purified from one another using standard techniques well-known to those skilled in the art.

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26. European Patent Publication 0138111 A1, published April 24, 1985, corresponding to European Patent Application No. 84111416.8, filed September 25, 1984, which claims priority of U.S. Serial No. 538,607, filed October 3, 1983, and U.S. Serial No. 609,412, filed May 11, 1984.

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Claims

1. A method of treating a subject suffering from a disorder associated with the generation of oxygen free radicals in quantities sufficient to cause undesirable symptoms which comprises administering to the subject an amount of human manganese superoxide dismutase, a human manganese superoxide dismutase analog or a human manganese superoxide dismutase mutant effective to alleviate the undesirable symptoms.

2. A method of claim 1, wherein the undesirable symptoms comprise inflammation.

3. A method of claim 2, wherein the inflammation is synovial inflammation.

4. A method of claim 3, wherein the human manganese superoxide dismutase, human manganese superoxide dismutase analog, or human manganese superoxide dismutase mutant is administered intraarticularly.

5. A method of claim 4, wherein the amount of human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is between about 1 and about 200 mg/joint.

6. A method of claim 1, wherein the disorder is arthritis and the undesirable symptoms comprise inflammation.

7. A method of claim 6, wherein the human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is administered daily.

8. A method of claim 7, wherein the amount of human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is between about 1 and about 100 mg/kilogram body weight of the subject.

9. A method of claim 8, wherein the amount of human manganese superoxide dimustase, human manganese superoxide dimustase analog or human manganese superoxide dimustase mutant is between about 3 and about 50 mg/kilogram body weight of the subject.

10. A method of claim 6, wherein the human superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is administered subcutaneously.

11. A method of claim 6, wherein the human superoxide dismutase, human manganese superoxide dismutase analog or human manganese supeoxide dismutase mutant is administered intravenously.

12. A method of claim 1, wherein the undesirable symptoms comprise lung fibrosis.

13. A method of claim 12, wherein the human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is administered daily.

14. A method according to claim 13, wherein the amount of human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manga nese superoxide dismutase mutant is between about 1 and about 100 mg/kilogram body weight of the subject.

15. A method of claim 14, wherein the amount of human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is between about 3 and about 50 mg/kilogram body weight of the subject.

16. A method of claim 12, wherein the fibrosis is induced by an anti-neoplastic agent.

17. A method of claim 12, wherein the fibrosis is induced by radiation.

18. A method of claim 16, wherein the anti-neoplastic agent is bleomycin.

19. A method of claim 12, wherein the human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is administered subcutaneously.

20. A method of claim 12, wherein the human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is administered intravenously.

21. A method of claim 12, wherein the human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant is administered intratracheally.

22. A double-stranded DNA molecule which encodes human manganese superoxide dismutase or a human manga nese superoxide dismutase analog and the coding strand of which has the nucleotide sequence shown in Figure 6.

23. A double-stranded DNA molecule which encodes a human manganese superoxide dismutase mutant and the coding strand of which has a nucleotide sequence which varies from the nucleotide sequence shown in Figure 6 by one or more nucleotides.

24. A cloning vehicle which comprises the DNA molecule of claim 22 or 23.

25. A cloning vehicle containing a fragment of the DNA molecule of claim 22 or 23.

26. A plasmid of claim 25, designated pMSG11-1 and deposited in Escherichia coli strain MC1061 under ATCC Accession No. 67363.

27. A plasmid of claim 25, designated pMSG4 and deposited in strain MC1061 under ATCC Accession No. 67364.

28. A plasmid of claim 25, designated pMSG-1b and deposited in Escherichia coli strain MC1061 under ATCC Accession No. 67365.

29. A eucaryotic cell in culture in which the DNA sequence of claim 22 or 23 is expressed.

30. A eucaryotic cell transformed with the cloning vehicle of claim 22 or 23.

31. A eucaryotic cell of claim 29 wherein the DNA is present in multiple copies.

32. A eucaryotic cell of claim 29, wherein the expression of the DNA is regulated by the regulatory elements associated with the noncoding DNA region of human manganese superoxide dismutase shown in Figure 6.

33. A mammalian cell of claim 29.

34. A human HeLa cell of claim 33.

35. A mouse L cell of claim 29.

36. A method for producing human manganese superoxide dismutase, human manganese superoxide dismutase analog, or human manganese superoxide dismutase mutant which comprises growing the eucaryotic cell of claim 29 under suitable conditions permitting production of the human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant and recovering the resulting human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant.

37. Human manganese superoxide dismutase, human manganese superoxide dismutase analog, or human manganese superoxide dismutase mutant produced by the method of claim 36.

38. A pharmaceutical composition comprising an amount of human manganese superoxide dismutase, human manganese superoxide dismutase analog or human manganese superoxide dismutase mutant effective to alleviate the undesirable symptoms associated with the generation of oxygen free radicals and a pharmaceutically acceptable carrier.

39. A human manganese superoxide dismutase analog in which at least one of the human manganese superoxide dismutase polypeptides differs from naturally-occurring human manganese superoxide dismutase polypeptide by the absence of lysine at the N-terminus.

40. A human manganese superoxide dismutase analog in which at least one of the human manganese superoxide dismutase polypeptides differs from naturally-occurring human manganese superoxide dismutase polypeptide by the absence of both lysine and histidine at the N-terminus.

41. A pharmaceutical composition comprising an amount of the analog of claim 39 effective to alleviate the undesirable symptoms associated with the generation of oxygen free radicals and a pharmaceutically acceptable carrier.

42. A pharmaceutical composition comprising an amount of the analog of claim 40 effective to alleviate the undesirable symptoms associated with the generation of oxygen free radicals and a pharmaceutically acceptable carrier.

43. A method of treating a subject suffering from a disorder associated with the generation of oxygen free radicals in quantities sufficient to cause undesirable symptoms which comprises administering to the subject an amount of the analog of claim 39 effective to alleviate the undesirable symptoms.

44. A method of treating a subject suffering from a disorder associated with the generation of oxygen free radicals in quantities sufficient to cause undesirable symptoms which comprises administering to the subject an effective amount of the analog of claim 40 effective to alleviate the undesirable symptoms.

Figure 1

```
          10        20        30        40        50        60
          •         •         •         •         •         •
GAA TTC GGC GGC GGC ATC AGC GGC TAA GGC AGC ACT AGC AGC ATG TTG AGC GGG GCA GTG
                                                Met Leu Ser Arg Ala Val

          70        80        90       100       110       120
          •         •         •         •         •         •
TGC GGC ACC AGC ACG CAG CTG GCT GGG GCT TTG GGG TAT CTG GGC TGC AGG CAG AAG CAC
Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala Leu Gly Tyr Leu Gly Ser Arg Gln Lys His

         130       140       150       160       170       180
          •         •         •         •         •         •
AGC CTC CCC GAC CTG GCC TAC GAC TAC GGC GGC CTG GAA CCT CAC ATC AAC GCG CAG ATC
Ser Leu Pro Asp Leu Pro Tyr Asp Tyr Gly Ala Leu Glu Pro His Ile Asn Ala Gln Ile

         190       200       210       220       230       240
          •         •         •         •         •         •
ATG CAG CTG CAC CAC AGC AAG CAC CAC GCG GCC TAC GTG AAC AAC CTG AAC GTC ACC GAG
Met Gln Leu His His Ser Lys His His Ala Ala Tyr Val Asn Asn Leu Asn Val Thr Glu

         250       260       270       280       290       300
          •         •         •         •         •         •
GAG AAC TAC CAG GAG GCG TTC GCC AAG GGA GAT GTT ACA GCC CAG ATA GCT CTT CAG CCT
Glu Lys Tyr Gln Glu Ala Leu Ala Lys Gly Asp Val Thr Ala Gln Ile Ala Leu Gln Pro

         310       320       330       340       350       360
          •         •         •         •         •         •
GCA CTG AAG TTC AAT GGT GGT GGT CAT ATC AAT CAT AGC ATT TTC TGG ACA AAC CTC AGC
Ala Leu Lys Phe Asn Gly Gly Gly His Ile Asn His Ser Ile Phe Trp Thr Asn Leu Ser

         370       380       390       400       410       420
          •         •         •         •         •         •
CCT AAC GGT GGT GGA GAA CCC AAA GGG GAG TTG CTG GAA GCC ATC AAA CGT GAC TTT GGT
Pro Asn Gly Gly Gly Glu Pro Lys Gly Glu Leu Leu Glu Ala Ile Lys Arg Asp Phe Gly

         430       440       450       460       470       480
          •         •         •         •         •         •
TCC TTT GAC AAG TTT AAG GAG AAG CTG ACG GCT GCA TCT GTT GGT GTC CAA GGC TCA GGT
Ser Phe Asp Lys Phe Lys Glu Lys Leu Thr Ala Ala Ser Val Gly Val Gln Gly Ser Gly

         490       500       510       520       530       540
          •         •         •         •         •         •
TGG GGT TGG CTT GGT TTC AAT AAG CAA CGG GGA CAC TTA CAA ATT GCT GCT TGT CCA AAT
Trp Gly Trp Leu Gly Phe Asn Lys Gln Arg Gly His Leu Gln Ile Ala Ala Cys Pro Asn

         550       560       570       580       590       600
          •         •         •         •         •         •
CAG GAT CCA CTG CAA GGA ACA ACA GGC CTT ATT CCA CTG CTG GGG ATT GAT GTG TGG GAG
Gln Asp Pro Leu Gln Gly Thr Thr Gly Leu Ile Pro Leu Leu Gly Ile Asp Val Trp Glu

         610       620       630       640       650       660
          •         •         •         •         •         •
CAG GCT TAC TAC CTT CAG TAT AAA AAT GTC AGG CCT GAT TAT CTA AAA GCT ATT TGG AAT
His Ala Tyr Tyr Leu Gln Tyr Lys Asn Val Arg Pro Asp Tyr Leu Lys Ala Ile Trp Asn

         670       680       690       700       710       720
          •         •         •         •         •         •
GTA ATC AAC TGG GAG AAT GTA ACT GAA AGA TAC ATG GCT TGC AAA AAG TAA ACC ACG ATC
Val Ile Asn Trp Glu Asn Val Thr Glu Arg Tyr Met Ala Cys Lys Lys ———

         730       740       750       760       770       780
          •         •         •         •         •         •
GTT ATG CTG ATC ATA GGC TAA TCA TCC CAG CAA GAT AAT GTC CTG TCT TCT AAG ATG TGC

         790       800       810
          •         •         •
ATC AAG CCT GGG TAC ATA CTG AAA GGC GGA AGT
```
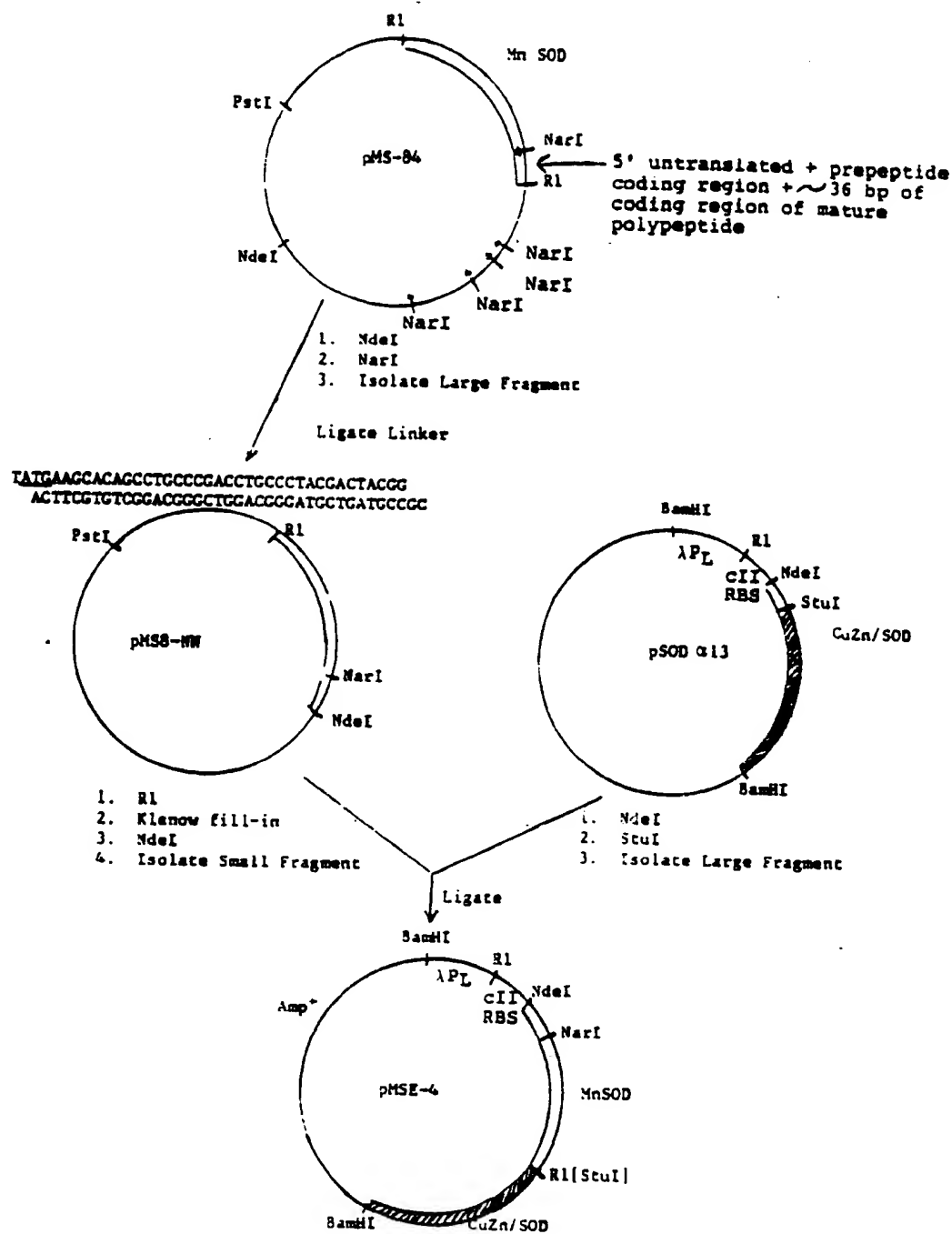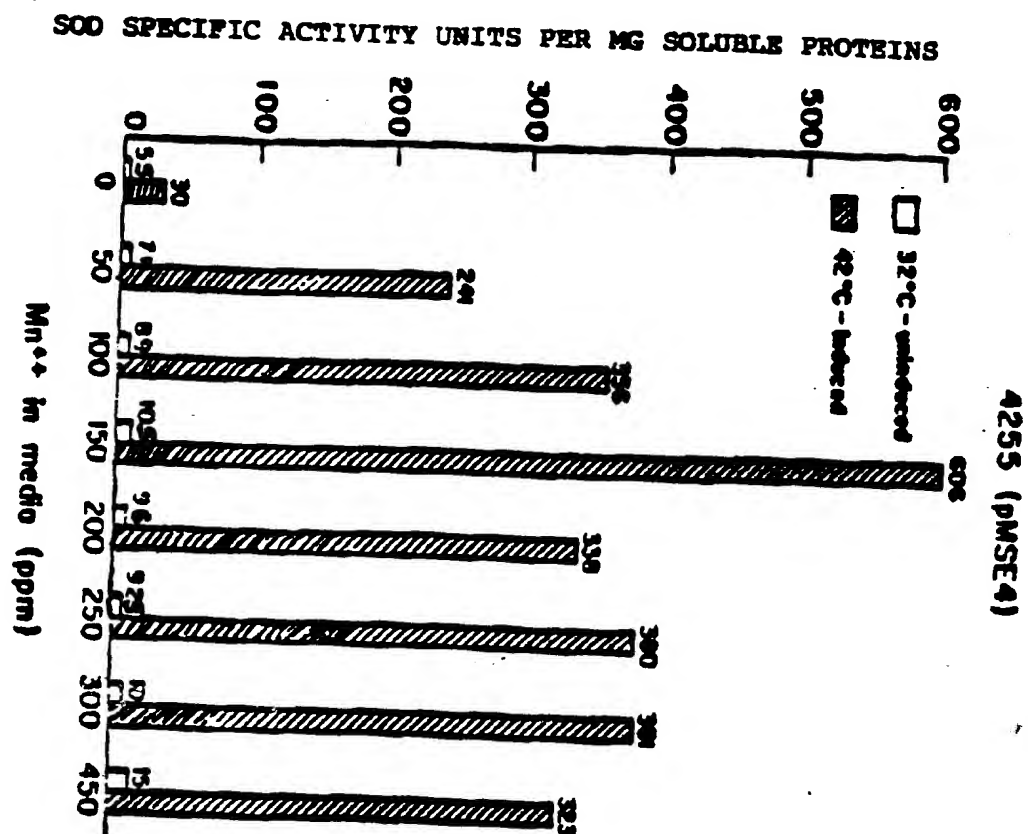
Figure 2

Figure 3

Figure 5

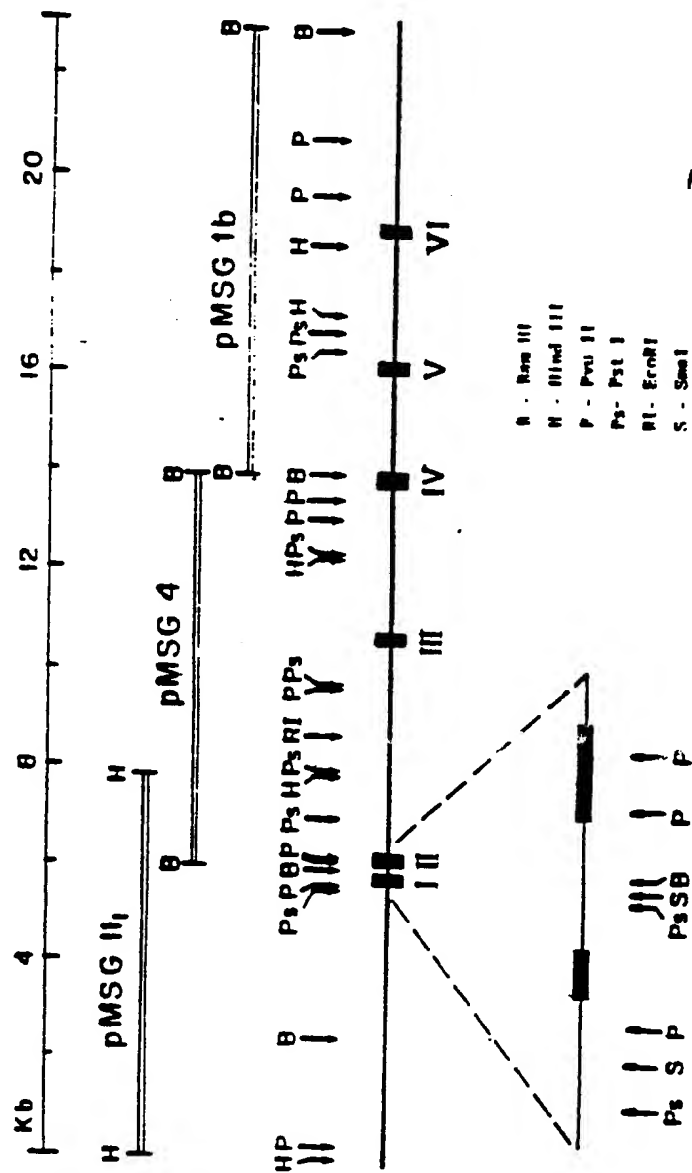Restriction map and organization of human MnSOD gene.

Figure 6

Nucleotide Sequence of Human MnSOD Gene

```
  1  AACCAAAAAC TCACGGGGCC AGCGCCGGCA GGGCCCCCTA GTGCAGCCAG ATCCCCGCCG
 61  GCACCTCAGG GGGCGGACCC GCAGGCAGGG CTTGCGGGCC GTACCAACTG CCACGGGGCC
121  AGGGGCCGCC TCCCTTCGGC CGCGCGCCAC TGCAAGTACG GCAGGACACC AGCGAGGTTG
181  CCGAGGCCGA GGCTAGCCTG CAGCCTCCTT TCTCCCGTGC CCTGGGCGCG GGGTGTACGG
241  CAAGCGCGGG CGGGCGGGAC AGGCACGGCA GGCACCGCCG GGGTTCGGGC GCGGCGGGGG
301  GGGGCGGGGC TCGCGGGGGG AGGGGGGCGG GCGGCGGTCG CCGTTGTCGG CGCAGCTGGG
361  TCGCGGCCGT CCTCCCGGCG CTTTCTTAAG GCCGGGGGGC GGCGCAGGAG CGGTCACTCG
421  TGCGCGTCGT CGGCCTCCGCA TCCGGCGTGG GCGGCATCGG CGGGCCGGCG GCCGCAGCGG
481  CTAGCAGCAT TGTTCAGCCG GCGACTGTCG GGTGAGAAGG AAGGGGACCC GGTCAGGCCC
541  CAAGGCGAAG GGGCTCGCGG CGGGCAGGGC GGTGAGAAGG AAGGGGACCC GGTCAGGCCC
601  CTGGCGCGAC CGCCGACCTG CAGCGCGGTTC TCCCGGGAGT GGCGGACATGG CCGCACGGGC
661  GCCGGGGATC CGAGGGGAGGG GACGCGGGGA CTCGGGCGGAC GCCGCGCTGC GTTCCTCGGC
721  AGCCCAGCCT GCGTAGACGG TCCGCGCGCC TGACTGCACCG GGCTGTGTCTT TCTCGTCTTC
781  AGGCACCAGG ...
841  CCTCCCCAG ...
901  GCAGCTGG ...
961  GAAGTAG ...
1021 AAAAAAATGT GGTTGCCACT TTTAACTTTT AAG...
1081 AGCGTGCAC ...
1141 TCAGCCCTAA ...
1201 CTTCTTGCAC AGTAGGAATC GTTGGATATA TTGTGCACCC TTATCTACAA
1261 GTAATTTCTT GGGCCCTATG GAT...
1321 GTAATTATTG GAAATCTGTT CATTTGTGG TTTTGAATAC ATGTAATATA ACATTTTACT
1381 ...
1441 ...
1501 ...
1561 CATTTAGATA GGGTTGAGTG TTGTTCCAGT TTGGAAAACG AGTCCACTAT TAAAGAAACGT
1621 GGACTCCAAC GTCAAAGGGC GAAAAACCGT CTATCAGGGC GATGGCCACT ACGTGAACCA
1681 TCACCCAAAT CAAGTTTTTT GGGGTCGAGG TGCCGTGAAA GCCACTAAATC GGAACCTAAA
1741 GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGGCGGGCG AACTGTCGGCG AGAAAGGGAAG
1801 GGAAGAAAGC GAAAGGAGCG GGCGGTAGGC GTGGCAATTG TGTAGCGGTCA CTGGGG...
1861 CCCCCTTCTT TCTAACAGGG ...
1921 ACTAGGTTCA GCTATAAAAT ...
1981 AGTGGTAGAA GTGTAAGTGAA ...
2041 TGAGTATGTT AAGCTCTTTA TGACTGACTA TGTAGTGGTA TAGAGTACTG CAG...
2101 GTCCATATCT AAAACCACGT ATAAACATTA AATTGTATTT CCTGTTTTAA TTCCAGGGGA
2161 AGTACTGTTT GGGAAAGCTA TTATTAGGTA AATGTTTTAC AAATTACTGT TTCTCAGTTT
2221 CAGTCATACC TGTAATGATCC CAGCAAGATA ATGTGCCTTC GTGTAGATGC GCCATCAAGG
2281 CTGGGTACAT AACTGAAACGC TATAAGGTCC CTGGCATAATT TTTGTTGAT TATTGCATTG
2341 AAGAAACATT TATTTTCCAA AATTGTGTGA AGTTTTTGAC TGTTAATAAA AGAATCTGTC
2401 TTCTGTCATT TTCACTGAGT TTCCATGCGA AAGGAATAGT TCATTAAAAA TCCTATAATC
2461 TATTACTCTT AAGACCAAGA CCTGTGTCTC CAGTCATATC TGTAATAACA TCATCTGATA
2521 ACCTAAAAGC ATAGTATTAG GGATATACGA CAAAACCAAA GTGTTTTTGC TGTTGTCACA
2581 TACCACTCAA TACTTTTACA CCAGTTTGTC CAGTGGGACT CCAGCTG...
```

Figure 7

Exon-Intron Junctions of Human MnSOD Gene

	exon	intron	exon
Intron # 1	TG TGCG	GGTGAG...CTTCAG	GCACCA
Intron # 2	CCAAGG	GTAGGT...TTTAAG	GAGATG
Intron # 3	CCAAAG	GTTGGA...TAATAG	GGGAGT
Intron # 4	CAACAG	GTTACA...TAACAG	GCCTTA
Intron # 5	TGCTGA	GTATGT...TTTCAG	TCATAC

		A	TT	
CONSENSUS	AG	GT AGT...	NCAG	G
		G	CC	

Figure 8

Pharmacokinetics of Mn-SOD After s.c. Injection Into Rats
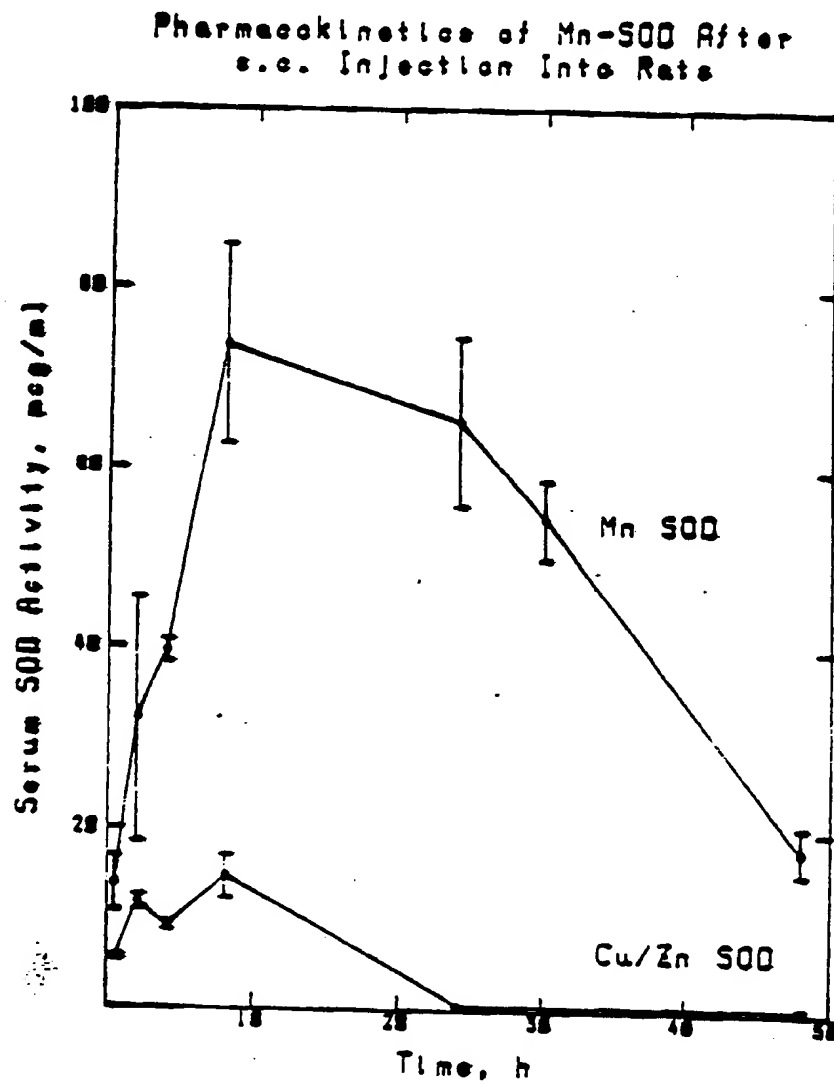
Figure 9

**EFFECT OF MnSOD ADMINISTERED 24 HOURS BEFORE INSULT
AND COMPARISON WITH CU/ZN SOD ANALOG ADMINISTERED
AT -2 HOURS AND -24 HOURS**

PAW EDEMA ASSAY   1/1/87

Figure 10

Pharmacokinetics of MnSOD in Rats

(1) -i.v 25 mg/kg Rat

(2) -i.p 25 mg/kg Rat

(3) -s.c 25mg/kg Rat
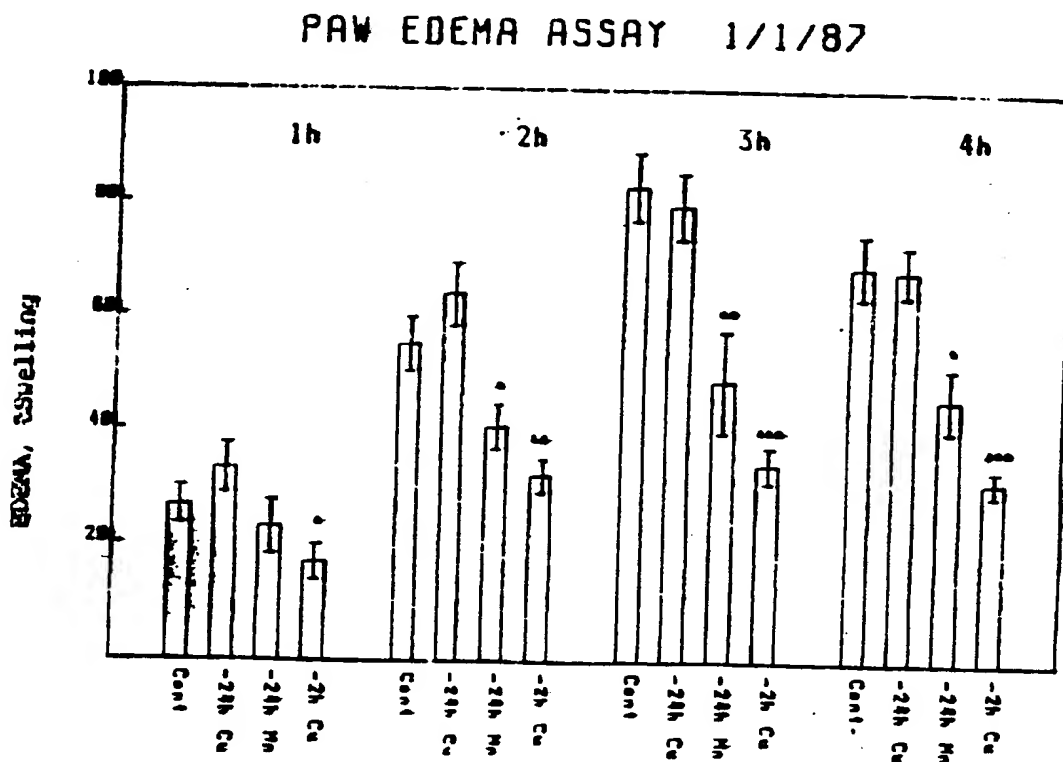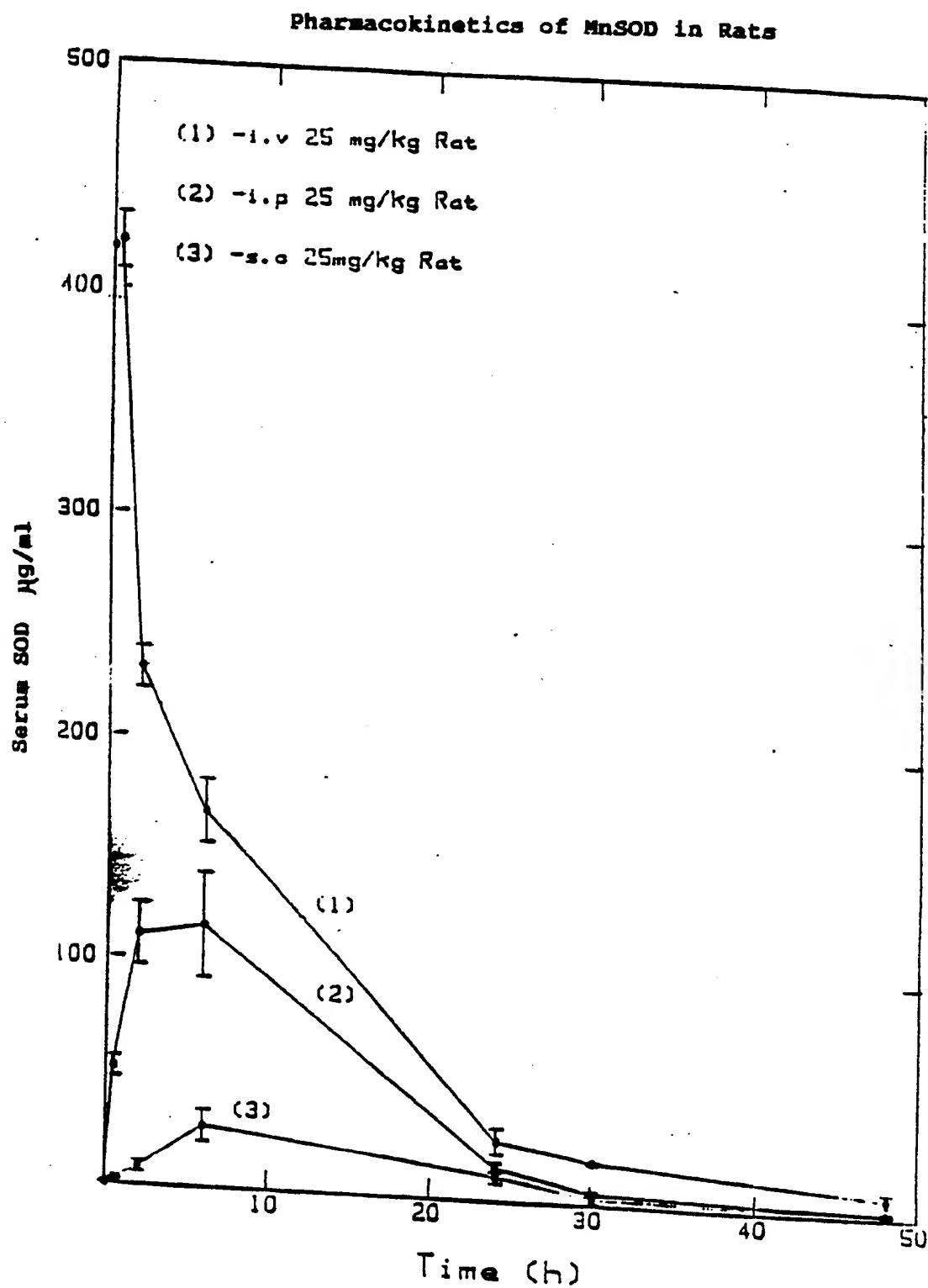
Figure 11

Effect of Intra-articular Cu/ZnSOD and MnSOD on Knee-Joint Inflammation

*   p<0.05
**   p<0.01
***   p<0.001

Synovial Weight mg
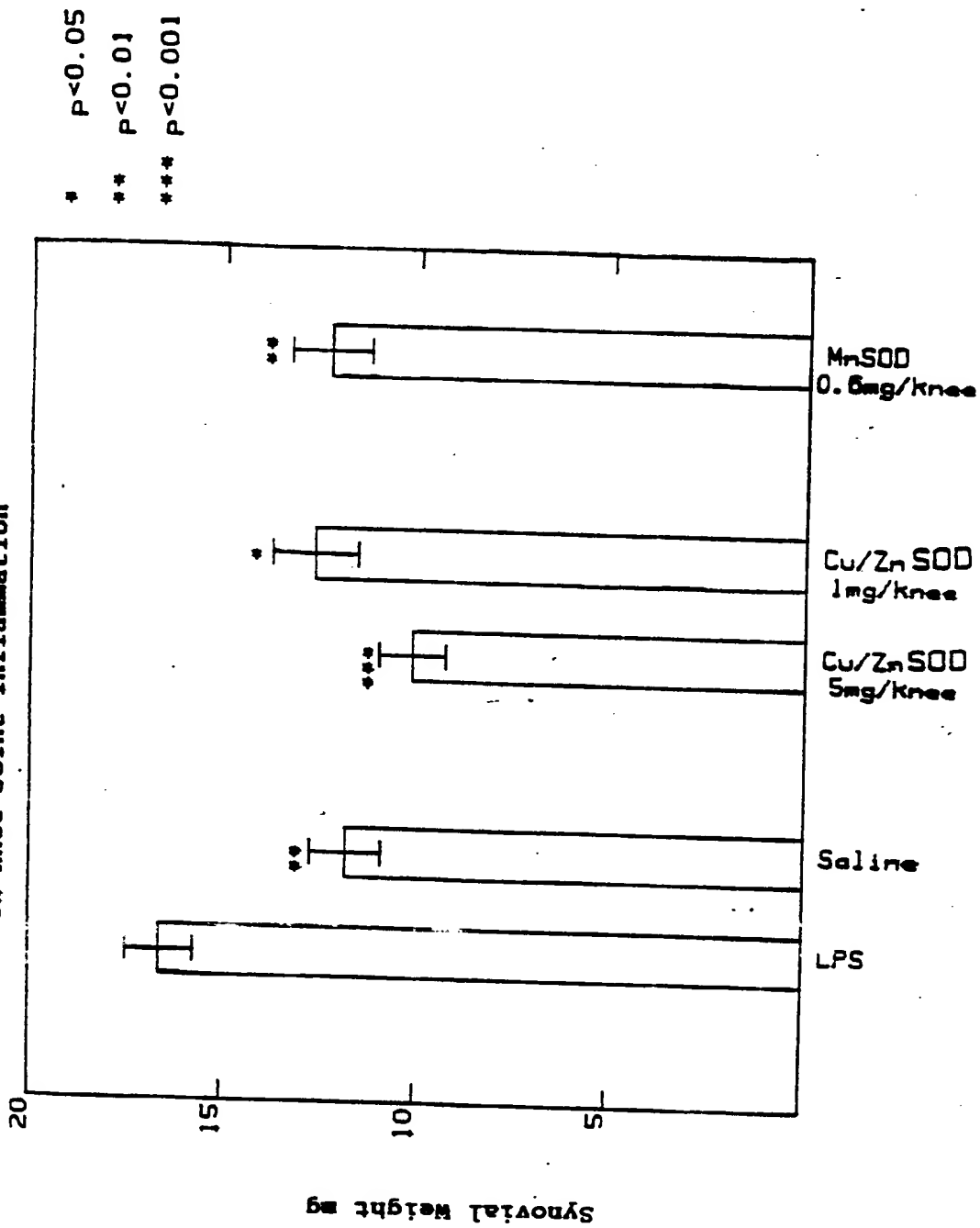
Figure 12

Effect of CuZnSOD and MnSOD on Adjuvant Arthritis
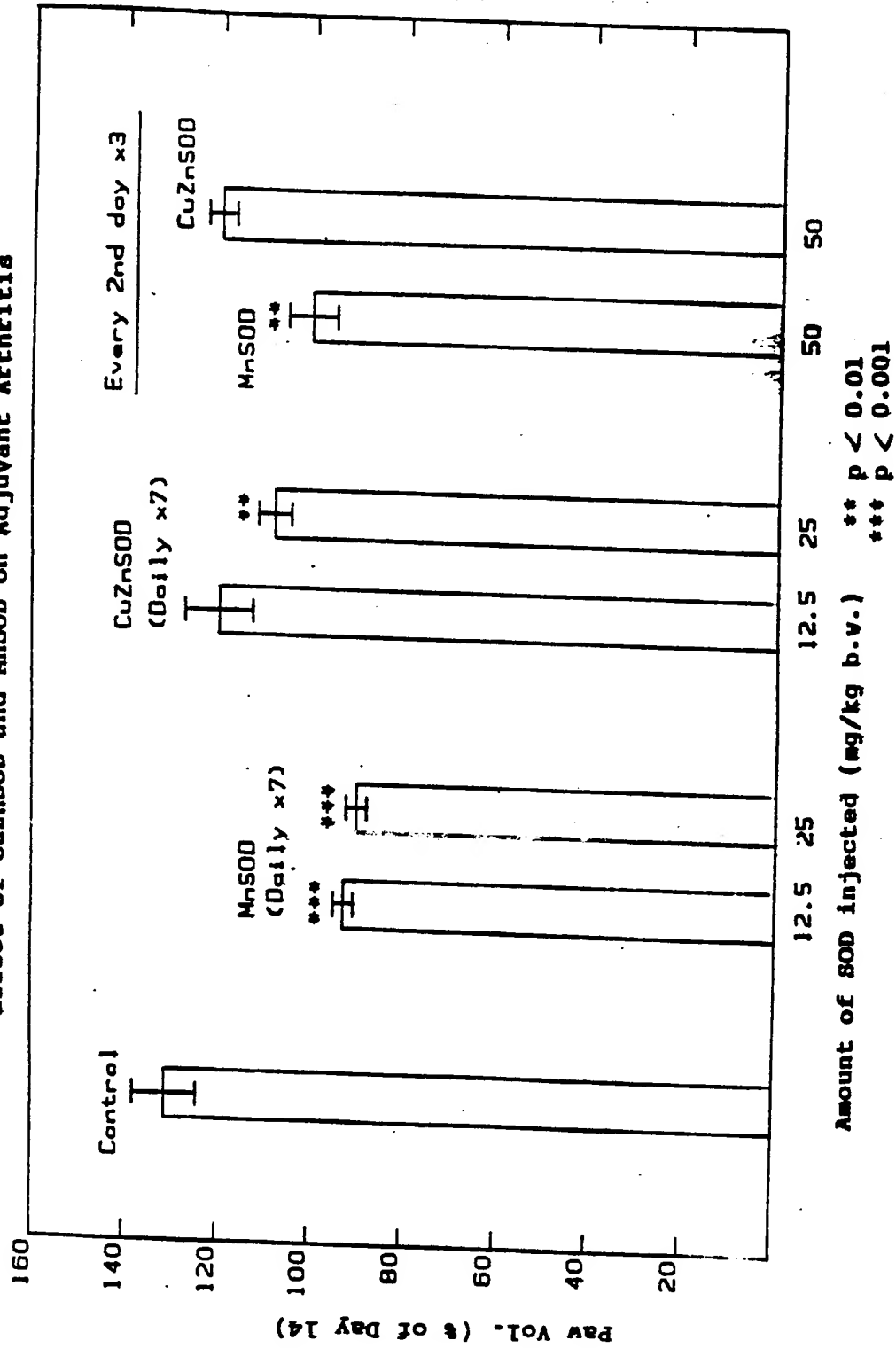
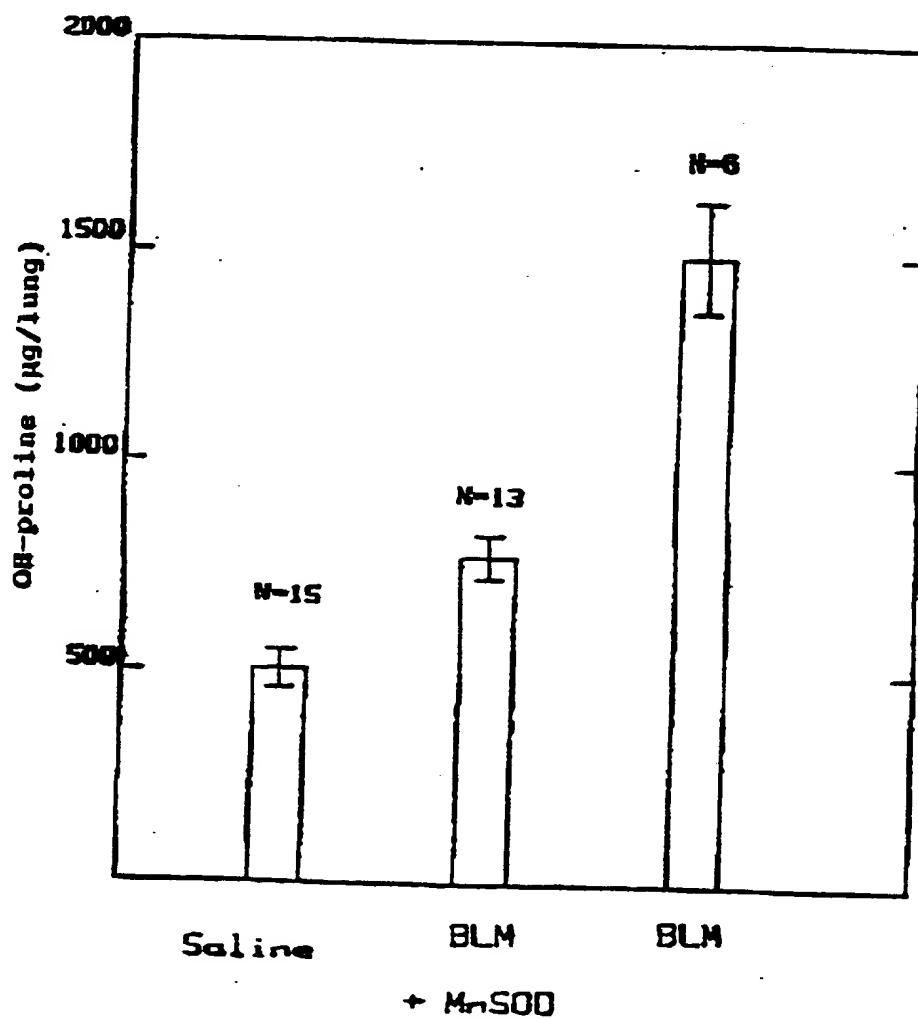Figure 13

Effect of MnSOD on Bleomycin-induced
Lung Fibrosis
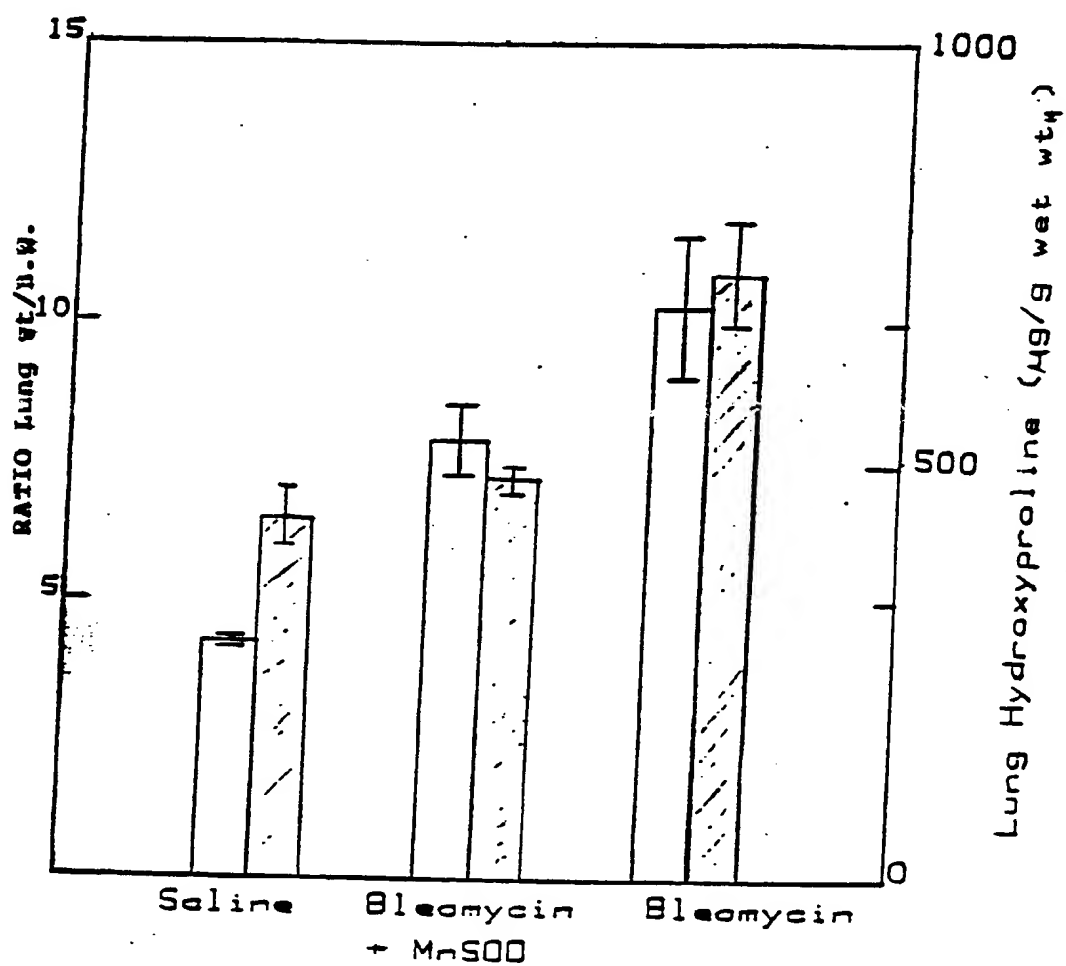
Figure 14

Effect of Daily S.C. Injections of MnSOD on Lung OH-Proline

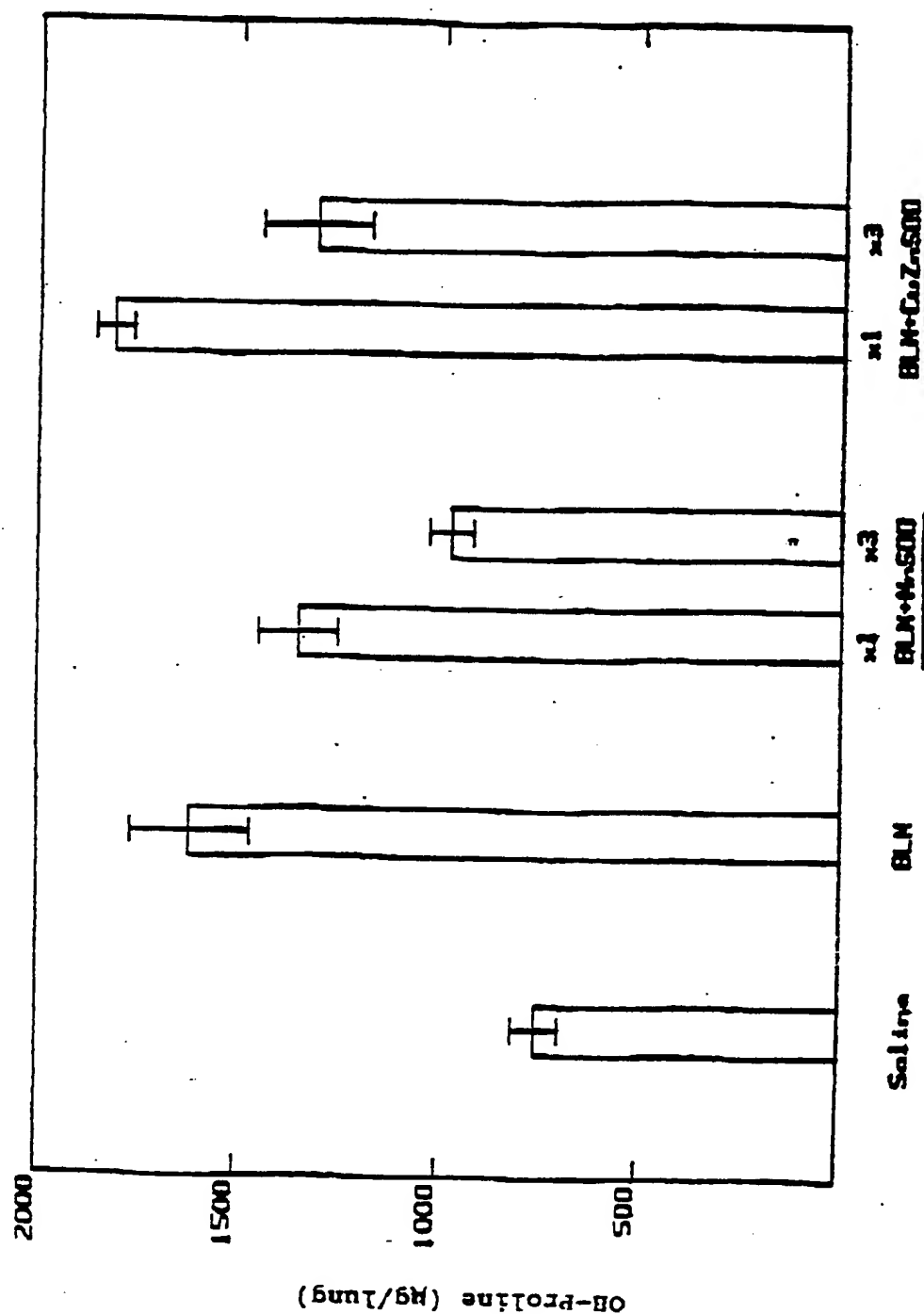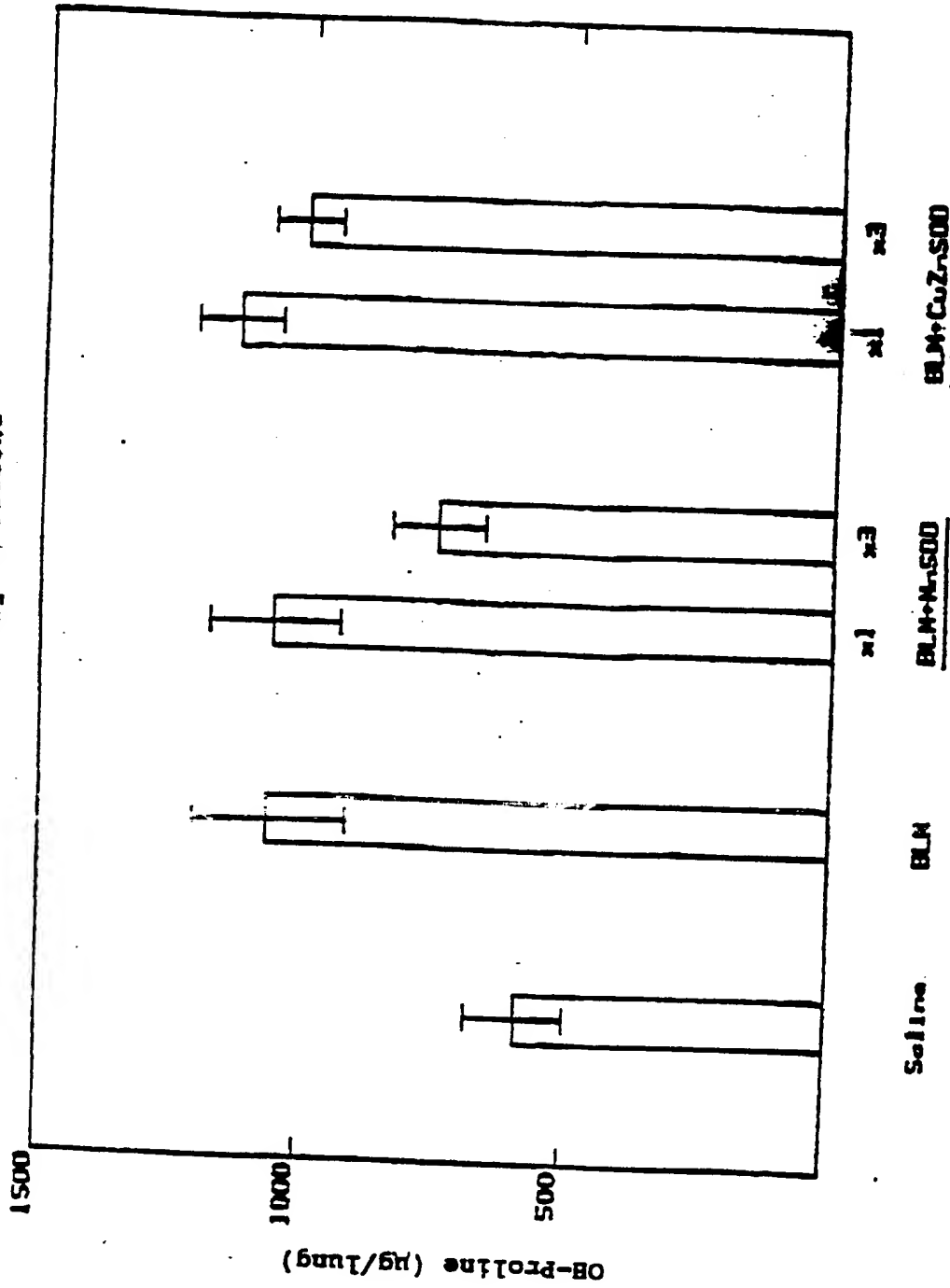Effect of S.C. Injections of MnSOD and CuZnSOD on Lung OH-Proline

Figure 15

Figure 16

Effect of I.P. Injections of MnSOD
and CuZnSOD on Lung OH-Proline

NOTE: x1 and x3 represent once a week or three times
a week, respectively.